

# **External Peer Review of the Draft Health Effects Support Document (HESD) for the Cyanobacterial Toxin Microcystins**

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Submitted to:

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## **Responses to Charge Questions**



**1. Chapters 2, 5, and 6 of the HESD provide information on the chemical and physical properties, exposure, and the toxicokinetics of microcystin.**

**1.1 Are you aware of any additional data that should be included in the document? If so, please provide.**

Reviewer	Comments	Response to Comments
<b>Chou</b>	None.	
<b>Hooser</b>	<p>a. 5.1.pg 23. Exposures from soil and edible plants: Data gap – Are microcystins bound in plants following uptake by the plant? After ingestion by mammals, are they available for binding tissues followed by toxicity in the person eating the plant?</p> <p>b. 5.2.pg.23. Exposures from fish and shellfish consumption: Data gap – same question as “a”, except are microcystins bound in seafood tissues following ingestion by the fish, shellfish, etc?”</p> <p>Q: Are there any documented cases microcystin toxicity in people or animals following ingestion of fish or shellfish that have ingested/been exposed to microcystins? Following ingestion of microcystins by fish or shellfish, the microcystins may be covalently bound to fish/shellfish protein and unavailable to cause toxicity in the people or animals eating them.</p> <p>Williams DE, Dawe SC, Kent ML, Andersen RJ, Craig M, Holmes CF. Bioaccumulation and clearance of microcystins from salt water mussels, <i>Mytilus edulis</i>, and in vivo evidence for covalently bound microcystins in mussel tissues. <i>Toxicon</i>. 1997 Nov;35(11):1617-25.</p> <p>Ibelings, BW, et al. Distribution of microcystins in a lake foodweb. No evidence for biomagnification. <i>Microbial Ecology</i> 49, 487-500, 2005.</p> <p>Website for Ibelings: <a href="http://www.unige.ch/forel/Ecologie-microbienne/Equipe/IbelingsB.html">http://www.unige.ch/forel/Ecologie-microbienne/Equipe/IbelingsB.html</a></p> <p>Dionisio Piers, L.M. Assimilation and depuration of microcystin-LR by the zebra mussel, <i>Dreissena polymorpha</i>. <i>Aquatic Toxicol.</i>, 69, 385-396, 2004.</p> <p>Dionisio Pires, L.M. ; Ibelings, B.W. ; Donk, E. van. Zebra mussels as a potential tool in the restoration of eutrophic shallow lakes, dominated by toxic cyanobacteria. In: Velde, G. Van der ; Rajagopal, S. ; Vaate, A.A. Bij de (ed.), <i>The Zebra Mussel in Europe</i>, pp. 361-372, 2009. Leiden: Backhuys Publishers.</p>	

	<p>John Fournie, Elizabeth Hilborn, Geoffrey Codd, Michael Coveney, Juli Dyble, Karl Havens, Bas Ibelings, Jan Landsberg, Wayne Litaker. Environmental Protection Agency Papers, Paper 37, Cyanobacterial Harmful Algal Blooms: Chapter 31: Ecosystem Effects Workgroup Report, 2008.</p> <p>Identifies some data gaps related to cyanobacterial toxins in the aquatic environment. It does not address the issue of bioavailability of microcystins to humans, mammals and birds eating fish and shellfish following uptake by those aquatic organisms.</p> <p>Data gap - 6.2 Distribution p29: Could not find a radiolabel study which described distribution of radiolabeled microcystin to all tissues accounting for 100% of label. In particular for this Health Effects Document, distribution to testis was not found in references. Most radiolabel (or immunohistochemical) studies that I had time to look up appear to leave out testis and ovaries.</p> <p>Q: Or, is it that testis or ovary were examined, but did not have any radioactivity or staining?</p> <p>6.2. Distribution, Oral, p31, 1<sup>st</sup> paragraph – MC-LR was not found in milk of dairy cattle that were exposed to <i>M. aeruginosa</i> cells via drinking water...MC-LR was not found in muscle of beef cattle fed <i>M. aeruginosa</i> cells either</p> <p>Orr PT, Jones GJ, Hunter RA, Berger K. Exposure of beef cattle to sub-clinical doses of <i>Microcystis aeruginosa</i>: toxin bioaccumulation, physiological effects and human health risk assessment. <i>Toxicon</i> 41, 613-620, 2003.</p> <p>Correction – p32, under, “Liver Tissues – <i>in vitro</i>”, 1<sup>st</sup> paragraph: The statement, “A study done in 1998 showed adverse effects in liver caused by MC (Theiss et al., 1988). As a result, many researchers have examined the distribution to the liver using cell cultures.”, is inaccurate. Well before this study, it was already well established that the liver was the major target organ for microcystin toxicity. Therefore, since in field and experimental instances of microcystin toxicity it was observed and established that the liver was the primary target organ, many researchers have examined the distribution to the liver using perfused liver and hepatic cell cultures.</p> <p>Clarification, p33, “Liver Tissues – <i>in vitro</i>”, 3<sup>rd</sup> paragraph, “Chong, et al. (2000) evaluated microcystin toxicity in eight permanent cell lines..., only two of which showed cytotoxicity following MC-LR exposure.” This is to be expected as the preceding paragraph explains that primary cultures of liver cells cease to express OATPs after being maintained in culture. If cultured cells of any type don’t have OATPs, then the amount of microcystin that makes it into the cells will be very small.</p>	
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	6.3 Metabolism – p34, 3 <sup>rd</sup> paragraph: Clarification - Microcystis toxin 7820 here and elsewhere refers to microcystin produced by <i>Microcystis aeruginosa</i> strain 7820. Strain 7820 primarily produced MC-LR. I do not recall if it produced any other microcystin congeners. In the 1980s, <i>Microcystis aeruginosa</i> strain 7820 was being cultured by Dr. W. Carmichael who provided the toxin to other researchers.	
<b>Manson</b>	I have conducted Medline and Google searches and have not identified any additional data that should be included in Chapter 2. Chapter 5 is beyond my technical expertise and I found the material difficult to read with often contradictory information. This Chapter could be reduced to emphasize areas where there are comparable data and consistent findings. Chapter 6 is well-written and the information appears to be complete.	
<b>Stump</b>	I am unaware of any additional data that should be included in this document.	
<b>Yu</b>	HESD has compiled all available information on the chemical and physical properties, exposure, and the toxicokinetics of microcystin. No additional data were found during the review period.	

**1.2 Is any of the information or conclusions included in the document incorrect, redundant or irrelevant? Please explain.**

<b>Reviewer</b>	<b>Comments</b>	<b>Response to Comments</b>
<b>Chou</b>	<p>For Chapter 5: p.23-27:</p> <p>p. 23, First paragraph, Line 8, “Cyanobacterial cells can bioaccumulate ...”: Should this be “Microcystins can bioaccumulate...”?</p> <p>p. 23, Paragraph 1, Line 8, “Cyanobacterial cells can bioaccumulate in zooplankton.... and as a result of grazing may settle out of water column leading to an accumulation in the sediment.”: Please use two separate sentences because bioaccumulation and accumulation in the sediment are two different things. Please clarify whether they are the cells or the toxins that are settled out of the water column.</p> <p>p. 23, second paragraph, Line 4-5: This mistake needs to be corrected. The data “3.23 ug/mg dw”, report by Codd et al. (1999), is a microcystin-LR equivalent level of 3 microcystins in the bloom and scum of the irrigation water supply, not a “cyanotoxin level detected in lettuce leaf extracts” as stated in Lines 4-5 of this paragraph.</p>	

	<p>p. 23, second paragraph, Line 8: Please delete the statement "... of little concern to human health." It is neither convincing nor informative. Please present concentrations found in plants and estimated level of exposure.</p> <p>Chapter 6, p.28-36:</p> <p>p. 29, paragraph 4: Adding following information can be helpful in the flow, i.e. building up the knowledge base for the readers:</p> <p>Microcystins compete with bile acid uptake at a transport system to enter hepatocytes (Thompson and Pace, 1992).</p> <p>p. 30, paragraph 3, "Covalent adducts of MC-LR, MC-LA, and MC-LL...": Which study demonstrated this?</p> <p>p.30, paragraph 4, Nisiwaki et al., 1994): Please state the dosages in ug/kg of bw.</p> <p>p. 30, paragraph 4, Nisiwaki et al., 1994: Please indicate that the i.p. dose is 1000 time higher than the oral dose. (Reviewer's explanation: The dose difference can affect the relative tissue distribution when the tissue or organ uptake depends on saturable or rate limiting transporters.)</p> <p>p. 30, paragraph 4, Line 4-5, "Small amounts of radiolabel...": Is this truly small amounts or small proportion, % of dose per organ or relative tissue concentrations? Please clarify.</p> <p>p. 30, paragraph 5, Line 2-3, "The tissue distribution...l": Is this relative amount (% of dose) or absolute concentration? Please clarify.</p> <p>p. 30, paragraph 5, Line 3-4, "Liver accumulation ...": which dose?</p> <p>p. 35, fourth paragraph, 4th-3rd to the last line: Is this sentence finished?</p> <p>p. 36, Third paragraph, (Falconer et al., 1986): What is the species?</p>	
<b>Hooser</b>	<p>5.1 and 5.2 p23-24 – The health risk to humans and animals by consumption of fish and shellfish depends not only on the bioaccumulation of toxins in edible fish tissue, but also the bioavailability of active toxin that is present and has sufficient activity to cause toxicity in the humans and animals.</p>	
<b>Manson</b>	<p>Some of the information in Chapter 5 is irrelevant and consideration should be given to reducing this Chapter to emphasize areas where there are consistent findings. Chapter 6 is complete but some of the information is redundant. It would be sufficient to describe the consistent findings without citing numerous studies that came to the same conclusion.</p>	

<b>Stump</b>	Chapters 2, 5, and 6 are well written. I did not find any incorrect, redundant or irrelevant information.	
<b>Yu</b>	Page 6 Line 1 Table 0-2 should be Table 2-2.	

**1.3 Please comment on the flow and continuity of these chapters and provide suggestions to enhance the utility of these chapters, if needed.**

<b>Reviewer</b>	<b>Comments</b>	<b>Response to Comments</b>
<b>Chou</b>	These chapters are well written.	
<b>Hooser</b>	Flow and continuity good.	
<b>Manson</b>	Reduce the technical detail in Chapter 5 and 6 to improve continuity and flow.	
<b>Stump</b>	I thought that the chapters were well written and provide very good background information for the hazard identification and dose-response chapters.	
<b>Yu</b>	<p>Page 11 line 18 “In marine systems, salinity gradients also induce stratification. As temperatures rise due to climate change, waters are expected to stratify earlier in the spring and the stratification will persist longer into the fall (Paerl and Otten, 2013b).” It is unclear what is the purpose of these sentences. There is no evidence to directly support that these changes have anything important to the microcystin.</p> <p>Page 27 Line 5 “In children, they have been used as an alternative, natural therapy to treat attention deficit hyperactivity disorders (ADHD).” It is unclear to me the rationale to mention the children and talk about ADHD. Is it related to the risk assessment of MC-LR?</p>	

## 2. Chapter 7 - Hazard Identification.

This chapter outlines toxicological studies, epidemiology, genotoxicity and mechanistic data. This chapter also includes the characterization of human health effects.

### 2.1 Are you aware of any additional critical studies for microcystins that should be included in the document? If so, please provide.

Reviewer	Comments	Response to Comments
Chou	None.	
Hooser	<p>Yes, there is no mention of the very critical studies describing case reports and analyses of serum and tissues from fatal and non-fatal human dialysis patients with liver damage that were exposed to microcystins in dialysis water that was obtained from surface water sources in Brazil. Although the patients in these cases had pre-existing disease, and exposure is intravenous rather than oral, the exposure to microcystins from surface water sources, presence of microcystins in serum and liver, and subsequent liver damage is clear and demonstrates the systemic effects of microcystin in humans. Many animal and <i>in vitro</i> studies, verified in many different laboratories support the distribution and uptake by the liver with subsequent hepatic damage which can be severe and fatal. These reports should be summarized in 7.1.2 Systemic Effects, or in a section of their own.</p> <p>A partial list of references to include and summarize:</p> <ol style="list-style-type: none"> <li>1. Carmichael WW, Azevedo SM, An JS, Molica RJ, Jochimsen EM, Lau S, Rinehart KL, Shaw GR, Eaglesham GK. Human fatalities from cyanobacteria: chemical and biological evidence for cyanotoxins. <i>Environ Health Perspect.</i> 2001 Jul;109(7):663-8.</li> <li>2. Jochimsen EM, Carmichael WW, An JS, Cardo DM, Cookson ST, Holmes CE, Antunes MB, de Melo Filho DA, Lyra TM, Barreto VS, Azevedo SM, Jarvis WR. Liver failure and death after exposure to microcystins at a hemodialysis center in Brazil. <i>N Engl J Med.</i> 1998 Mar 26;338(13):873-8.</li> <li>3. Hilborn ED, Soares RM, Servaites JC, Delgado AG, Magalhães VF, Carmichael WW, Azevedo SM. Sublethal microcystin exposure and biochemical outcomes among hemodialysis patients. <i>PLoS One.</i> 2013 Jul 24;8(7):e69518. doi: 10.1371/journal.pone.0069518. Print 2013.</li> <li>4. Hilborn ED, Carmichael WW, Soares RM, Yuan M, Servaites JC, Barton HA, Azevedo SM. Serologic evaluation of human microcystin exposure. <i>Environ Toxicol.</i> 2007 Oct;22(5):459-63.</li> </ol>	

	<p>5. Soares RM, Yuan M, Servaites JC, Delgado A, Magalhães VF, Hilborn ED, Carmichael WW, Azevedo SM. Sublethal exposure from microcystins to renal insufficiency patients in Rio de Janeiro, Brazil. <i>Environ Toxicol.</i> 2006 Apr;21(2):95-103.</p> <p>6. Hilborn ED, Carmichael WW, Yuan M, Azevedo SM. A simple colorimetric method to detect biological evidence of human exposure to microcystins. <i>Toxicon.</i> 2005 Aug;46(2):218-21.</p> <p>7. Azevedo SM, Carmichael WW, Jochimsen EM, Rinehart KL, Lau S, Shaw GR, Eaglesham GK. Human intoxication by microcystins during renal dialysis treatment in Caruaru-Brazil. <i>Toxicology.</i> 2002 Dec 27;181-182:441-6.</p> <p>8. Pouria S, et al., Fatal microcystin intoxication in haemodialysis unit in Cararu, Brazil. <i>Lancet</i> 352, 21-26, 1998.</p>	
<b>Manson</b>	I have conducted independent literature searches and have not found any additional critical studies that should be included in the document.	
<b>Stump</b>	I am unaware of any additional studies that should be included in this document.	
<b>Yu</b>	<b>Page 58, Line 12 of the paragraph 2</b> “Histologically both treatment groups had atrophy of the seminiferous tubules with increased spacing between the seminiferous tubule cells. The effect increased with increasing dose. The high-dose group also exhibited deformation of androgonial and sperm mother cells, and decreased number of interstitial cells, Sertoli cells, and mature sperm in the seminiferous tubules.” It is unclear what is “androgonial and sperm mother cells”. It should be explained using the updated terminology.	

**2.2 Is any of the information included in the document incorrect, redundant or irrelevant? Please describe and provide suggestions, if needed.**

<b>Reviewer</b>	<b>Comments</b>	<b>Response to Comments</b>
<b>Chou</b>	<p>For Chapter 7, p.37-98:</p> <p>p. 37, Paragraph 1, the months of June through September...”: What year?</p> <p>p. 46, Fitzgeorge et al. (1994): Please add the following info for the study by Fitzgeorge et al. (1994): This study used newly weaned CBA/Balbc mice weighing 20 g (+/- 1g). Sex of the</p>	

	<p>mice and the number of mice per group in the tests for LD50 were not reported. Deaths were recorded within 2 hrs of dosing.</p> <p>p. 46, paragraph 4: Please specify the age of the “aged” mice.</p> <p>p. 47, Fitzgeorge et al. (1994): Please consider adding the following information:</p> <p>This study used newly weaned CBA/Balbc mice, 6 per treatment group, and sex was not specified. Deaths were recorded for 2 hours after a single dose. The estimated LD50 of intranasal instillation, 250 ug/kg, is the same as the LD50 of I.P. exposure, which is much lower than the LD50 of gastric intubation (3000 ug/kg). Aerosol inhalation of 0.005 ug/kg resulted in no death. A single LD50 dose, regardless of the route of exposure resulted in approximately 45% of liver weight increase. A higher liver weight increases (87%) was observed after the single i.n. dose of 500 ug/kg. While a single i.n. dose of 31.3 ug/kg had no effect on liver weight, repeat doses of i.n. 31.3 ug/kg, once a day for seven days, resulted in a 75% increase in liver weight.</p> <p>p. 48, Huang et al. (2011), Line 2: Please correct mistake. “Groups of 5 <u>mice</u>....”</p> <p>p. 52, Fawell et al. (1999): Please indicate that, in addition to “age not specified”, body weight is not specified. <u>Reviews comment</u>: This is unfortunate because Body weights and liver weights are important measurements in this study. Initial body weights could have been used to approximate age.</p> <p>p. 52-53, Fawell et al. (1999), “Mean body weight gain was decreased approximately 15% in all treated male groups.” This amount of decrease in mean body weight gain should not be dismissed when considering NOAEL. Liver weight is not reported in the publication.</p> <p>p. 56, Kirpenlo et al. (1981), “Changes in the estrous cycle...”: “Absence of estrus cycle...” is a more specific description. Absence of estrus cycle is reported on p.265 of the cited article.</p> <p>p. 56, Kirpenlo et al. (1981): Please clarify that the increase in primordial follicles, the decrease in mature follicles, the degeneration of oocytes in Graafian vesicles, the decrease in follicle dimensions, and the increase in the number of involuted corpora lutea were observed after 1.5 months of treatment, while the absence of estrus cycle and atrophy of uterus and genital appendages were observed after 3 months of treatment.</p> <p>p. 56, Kirpenlo et al. (1981), “Effects on Sertoli cells and spermatogonia were also noted.”: Please clarify. “Morphological abnormalities in Sertoli cells and degenerating spermatogonia were also noted.”</p>	
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	<p>p. 56, Kirpenlo et al. (1981), A note from the Review: The article is reviewed in details because it is an important study that supports the report by Chen et al. (2011). Unfortunately, the test substance used by Kirpenlo et al. is significantly different from that by Chen et al.</p> <p>p. 57, Falconer et al. (1988). Please state that the parental mice, 8 females and 2 males, at the age of 20 weeks, received the treatment for 17 weeks before mating.</p> <p>p.57, Paragraph 3, Line 6, Liu et al., 2010, "...Sertoli cells, were seen in animals treated with...": Please consider additional info on age. "...Sertoli cells, were seen in immature male Japanese White Rabbits (1.6+/- 0.2 kg) treated with..."</p> <p>p. 60, Ito et al. (1997b): What is the sex and age of the mice?</p> <p>p. 62, Paragraph 3, Zhang et al. (2012)"Body weight results were not reported": Body weight results are reported/described in the text of the Supporting Information (p.4), although data are not shown.</p> <p>p. 62, Paragraph 3, Zhang et al. (2012), "...infiltrating lymphocytes...(doses not specified)." Results of does related infiltrating lymphocytes are provided in Supporting Information in text (p.4) and in Figures S1B and S1C.</p> <p>p. 63, Please cite reference in the first sentence of Paragraphs 2, 3, and 4.</p> <p>p. 78, Paragraph 3, "The cell-type specificity of microcystins was investigated using...": Do you mean "The cell-type specificity of microcystin effects was investigated using..."? </p> <p>p. 82, Paragraph 2, "Increases in MDA....administered crude extracts...by Li et al. (2011b)": please indicate routes of exposure.</p> <p>p. 92, Paragraph 3, "... inhibiting their function (Craig et al., 1996)", "... inhibiting their functions (Craig et al., 1996)".</p> <p>P. 97-98, "Potentially Sensitive Populations": The assertion that "There are gender differences for reproductive effects..." has no supporting data. The i.p. dose of 5 ug MC-LR/kg in mice is an effective dose on serum level of progesterone (Wu et al., 2014), and no dose lower than this has been tested.</p>	
Hooser	<p><b><u>7.1 Human Effects</u></b></p> <p><u>7.1.1 Epidemiological Studies p37</u></p> <p>The Executive Summary on p2 adequately summarizes the limitations of the epidemiological studies that are presented in this section.</p>	

	<p>Zhou et al., 2002: Table 7-1, p38, Relative Risk of Colorectal Cancer and Microcystin Concentration by Drinking Water Source.</p> <p>As explained in the paragraph below it beginning, “This study provides suggestive...,” the title of this table is misleading and because of its prominent placing should be changed to, “Relative Risk of Colorectal Cancer by Drinking Water Source.”</p> <p>Figure 7-1, p39. Similarly, the title of this figure is also misleading because it gives the impression that there is a definitive relationship between colorectal cancer and microcystin exposure when the summary on p38 explains that the study may not have been adequately controlled.</p> <p><u>7.1.2 Systemic Effects</u></p> <p>Pg41 Turner et al., 1990. It should be added that this brief description of two cases notes that the fevers and clinical symptoms in the two army recruits resolved following administration of antibiotics, and serum liver enzyme activity was measured and was normal. Therefore, although this brief case report is used to support adverse pulmonary effects, of microcystins through inhalation, it does not support it and I doubt that the clinical symptoms reported were due to microcystin exposure.</p> <p>Pg42 Falconer et al., 1983. For comparison to Turner, et al., this paper by Falconer, et al. describes a report of illness in a population who were exposed to a <i>Microcystis aeruginosa</i> bloom via drinking water and suffered liver damage as measured by increases in liver enzyme activity in part of the population. Although not definitive, the symptoms and increases in liver enzyme activities are supported by a large body of experimental studies showing that microcystins cause liver damage, and by the human cases in Brazil in which patients were exposed to microcystin in dialysis water.</p> <p><u>Omission of important studies</u> - There is no mention of the very important studies describing case reports and analyses of serum and tissues from fatal and non-fatal human dialysis patients with liver damage that were exposed to microcystins in dialysis water that was obtained from surface water sources in Brazil. Although the patients in these cases had pre-existing disease, and exposure is intravenous rather than oral, the exposure to microcystins from surface water sources, presence of microcystins in serum and liver, and subsequent liver damage is clear and demonstrates the systemic effects of microcystin in humans. Many animal and <i>in vitro</i> studies, verified in many different laboratories support the distribution and uptake by the liver with subsequent hepatic damage which can be severe and fatal. These reports should be summarized in 7.1.2 Systemic</p>	
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	Effects, or in a section of their own.	
<b>Manson</b>	There is information provided in this Chapter which may be considered redundant or irrelevant for hazard/risk assessment. The sections on protein phosphatase inhibition (p. 86), cytoskeletal disruption (p. 89), apoptosis (p. 92) and reactive oxygen generation (p.94), while critical for hazard assessment, can be written in a much more concise manner.	
<b>Stump</b>	<p>I have identified a few errors.</p> <p>p.49, last sentence – The low dose should be 50 µg/kg/day, not 50 mg/kg/day.</p> <p>p.56, 3<sup>rd</sup> sentence – The author states that that the mid and high dose groups had a trend towards higher FSH after 3 months which reached statistical significance by 6 months. This is only true for the high dose group. Statistical significance was not achieved in the mid dose group for FSH at 6 months.</p> <p>p.56, 1<sup>st</sup> paragraph, last sentence – The LOAEL should be 0.79 µg/kg/day, not 0.79 mg/kg/day.</p> <p>p.58, 3<sup>rd</sup> paragraph – The author does not list the route of administration in the description of the Li et al. study.</p>	
<b>Yu</b>	<p>Page 89, line 27 “A study in China evaluated liver damage in children in relation to the microcystin levels in the drinking water and select aquatic foods (e.g., carp and duck) (Li et al., 2011a). Microcystin levels were associated with increasing levels of AST and ALP, but not ALT and GGT. The OR for liver damage as reflected by increased serum enzyme levels in exposed children was 1.72 (95% CI: 105-2.76).” I would like to suggest to use “increasing level of AST and ALP” instead of “liver damage”. Increase of ALP or AST does not mean the damage of the liver. Other diseases or factors can also cause the increase of ALP. The normal range of male and female is significantly different, males from 14-20 U/L, and female from 10 to 36 U/L. The results of this study did not separate the gender. It seems that all the values for the AST and ALP were within the normal range. Therefore, it is inappropriate to conclude that microcystin exposure led to liver damage.</p> <p>Page 90, Line 30 “Evidence for effects of MC-LR on the male reproductive system and sperm development following oral exposures were reported by Chen et al. (2011) and are supported by i.p. data (Liu et al., 2010; Chen et al., 2013). Oral exposure and i.p treatment are significantly different, especially for microcystin. Microcystin-LR is 30 ± 100 times less toxic via oral ingestion than via intraperitoneal injection (Fawell et al., 1999). The changes of male reproductive functions in i.p. did not support that oral exposure at low concentration could also</p>	

	<p>result in dysfunction of the male reproductive system and sperm development. It should be revised.</p> <p>Page 90, line 36, “deformation of androgonial and sperm mother cells;” should be updated using most recent terminology.</p> <p>Page 91, Line 3, it should add that microcystin (MC-LR) affects hormones level of male mice by damaging hypothalamic-pituitary system, but MC-LR was not able to enter Leydig cells and had no cytotoxicity on Leydig cells in vivo test. These results suggested that MC-LR affected male mice serum hormones and mRNA expressions by damaging the hypothalamic-pituitary systems (Wang et al., 2011).</p> <p>Page 91 line 4, It is really too sudden to follow the paragraph “The concerted action of protein phosphatases and kinases regulating the phosphorylation of the cytoskeleton is known to be important to sperm physiology. In a study of human normozoospermic and asthenozoospermic samples, Fardilha et al. (2013) identified a significant decrease in the cellular distribution of the PP1 and PP2 subfamilies that correlated with the low motility for the asthenozoospermic samples. The progressive motility of sperm in the asthenozoospermic samples was about 10% of that for the normal sperm and the number of immotile sperm was about twice that for the normal samples. Fardilha et al. (2013) is not a study of microcystins but gives credibility to the hypothesis that inhibition of protein phosphatases can adversely impact sperm motility.” There was no any paragraph discussing “that inhibition of protein phosphatases” is the mechanism of MC-LR induced-dysfunction of the sperms. There was no any discussion of the hypothesis. It is weird that this cited paper gave the credibility of the hypothesis. It is very critical to make clear what is the hypothesis, and who proposed the hypothesis.</p> <p>Page 91 Line 16 “Observed effects in <i>in vivo</i> studies include decreased sperm motility, viability, and counts; reduced spermatogonia and spermatid quality; and increases in abnormal sperm (Ding et al., 2006; Li et al., 2008). Numerous histological changes have also been observed in the testes including testicular atrophy and degeneration; depopulation of the Leydig, Sertoli, and mature sperm cells; and increased apoptosis (Ding et al., 2006; Li et al., 2008).” Reduced spermatogonia was not a significant change. In fact, there was no very robust study to demonstrate it. “Numerous histological changes” were not well justified by reviewing the publication. The most frequent histopathological observations were suffering from the artifacts of the fixation of the testis. Increased empty spaces between the seminiferous tubules were observed in the most of the histopathological examinations (Chen et al., 2013). Unfortunately, it was also observed in the most of the control from the representative photos since it was</p>	
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	<p>due to the inappropriate fixation of the testis tissue. It seems that the decrease of the Leydig cells was consistently observed, but no robust quantitative analysis to support it.</p> <p>Page 92, Line 7 from bottom, “The damage observed in each of the tissues impacted by microcystins (liver, testes, kidney, etc.) can be correlated with the mode of action events described above. The adverse effects observed are consistent with the postulated mode of action as are the dose-related increases in effect severity” Please describe clearly what is the “mechanistic mode of action of MC-LR induced adverse effects”. Simply listing of the reported changes of OATp transporter, phosphatase inhibition, cytoskeleton or the generation of ROS does not guarantee these observed changes are the MOA of MC-LR induced adverse effects in the target tissue of the liver or male reproductive systems. Again, it is unclear to me what is the postulated model of action.</p> <p>Page 98, line 1 to 5, “Available information does not suggest any pronounced gender differences in response to microcystins for the liver. Studies with cyanobacterial extracts suggest the possibility that male mice may be more sensitive than female mice to oral exposure to cyanobacterial extracts (Falconer et al., 1988). There are gender differences for reproductive effects as a consequence of sperm count, sperm motility, abnormal sperm, and histological alterations observed in the testes.” The above conclusions are misleading. Studies with cyanobacterial extracts were focusing on the male reproductive system. There were very few studies focusing on the effect on the female reproductive system. Based on the consistent effects of the hormonal changes (FSH, LH) and potential targets on hypothalamic-pituitary systems, exposure to female animals might result in changes of estrous cycle, and ovulation. There is a lack of information regarding the sensitivity in the reproductive system. The majority of the studies published in the male reproductive system do not mean that male reproductive system is more sensitive than the female reproductive system.</p>	
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**2.3 Are the conclusions and critical discussions for microcystins valid and scientifically defensible?  
Please describe and provide suggestions, if needed.**

Reviewer	Comments	Response to Comments
<b>Chou</b>	<p>P. 55, Chen et al. (2011): Methanol was present in the treatment doses, but not in the control's. Methanol has known effects on testosterone, FSH and LH (Cooper et al., 1992). Furthermore, synergistic effect of methanol with nutrient and aging factors on testicular function has been demonstrated by (Lee et al., 1991). see references below this paragraph. According to Chen et al. (2011), the MC stock solution was 1 g MC-LR per L of 0.1% methanol solution. The exposure to methanol in the treated</p>	

	<p>animals would be in the same range as the dose of MC-LR, if the reported 0.1% of methanol was a weight to volume expression. On the other hand, the 0.1% is more likely a volume-to-volume expression, therefore, the amount of methanol in the final treatment dosages would be about 80% of the MC-LR doses. These doses are about 1000 times lower than an effect dose of methanol in mice. (The reviewer realized later that this weakness in study design has been discussed on p.102, but believes that it should be stated in this part of the Draft.)</p> <p>Cooper RL, Mole ML, Rehnberg GL, Goldman JM, McElroy WK, Hein J, et al., 1992. Effect of inhaled methanol on pituitary and testicular hormones in chamber acclimated and non-acclimated rats. <i>Toxicology</i> 71(1-2): 69-81.</p> <p>Lee E, Brady AN, Brabec MJ, Fabel T. 1991. Effects of methanol vapors on testosterone production and testis morphology in rats. <i>Toxicology and industrial health</i> 7(4): 261-275.</p> <p>p. 58, Li et al. (2008): Methanol was present in the treatment doses, but not in the control's. See reviewer's comment above for p. 55, Chen et al. (2011), on methanol. In an effort to examine potential interactions between methanol and MC-LR, the reviewer compared the dose-response relationship of the i.p. route of exposure to MC-LR with methanol (Li et al., 2008) with the same i.p. dosage without methanol (Chen et al., 2013). The former study was conducted in Sprague-Dawley rats of 90-120g for 28 days, and the latter in Wistar rats of 180-200g for 50 days. The decrease in testicular weight was observed in both, although only statistically significant in the latter study. In addition, abnormality of seminiferous tubules was observed in the 5 ug/kg dose group in the former study and it too was observed in the 1 ug/kg group of the latter study. These comparisons indicate that at these dosages there are probably no apparent synergistic effects of methanol and MC-LR on testicular injuries. Such a comparison of the dose-response relationships of two i.p. studies and the resultant interpretation may alleviate some of the concerns over the presence of methanol in the treatments applied by Chen et al. (2011), but no interpretation can be used to dismiss the fundamental mistake in the study design by Chen et al. (2011). Please note that the authors in Chen et al. (2013) do not overlap with those in Chen et al. (2011) and Li et al. (2008).</p>	
<b>Hooser</b>	<p><u>7.2.5 Developmental/Reproductive Toxicity</u>  <u>Reproductive Effects</u>  <u>Oral</u></p> <p>Chen et al., 2011, p55. Paragraph beginning, "Sperm quality..." It should be made clear that the manuscript by Chen et al., 2011 did not include the calculations for estimation of oral dose in</p>	

	<p>the mice in this study. These calculations were made after the fact, presumably by those preparing this summary. It would be more appropriate to move these calculations to section 8.1.1, RfD Determination.</p> <p>Kirpenko et al., 1981, pg 56 – This reference is from a non-peer reviewed study published as a book chapter. I could not find an associated peer-reviewed, published manuscript. This study was performed with a natural population of what is reported to be <i>M. aeruginosa</i> at a time prior to purification, identification and chemical characterization of MC-LR. Therefore, while interesting and worthy of follow-up studies, this study itself does not provide support for reproductive or developmental effects of microcystin.</p> <p>Falconer, 1988 – This reference should be Falconer, et al. (1988). While this reference is from a peer-reviewed manuscript, it too utilized a bloom of what is reported to be <i>M. aeruginosa</i> at a time prior to purification, identification and chemical characterization of MC-LR. For the most part, it contradicts the studies of Kirpenko, 1981 and Chen, 2011. Therefore, it indicates a need for further reproductive and developmental studies.</p> <p><u>Other Routes</u></p> <p>Li et al. (2011), Chen et al. (2013), Li et al. (2008), and Wu et al. (2013) are all out of the same laboratory (X. Han). The only study from another laboratory is the study by Ding et al. (2006) (p58). This study used a crude extract from a <i>Microcystis sp.</i> bloom which is shown to contain microcystin LR as one of its components. This study is suggestive of a reproductive effect of <i>Microcystis sp.</i> bloom material, but does not specifically identify microcystin as the reproductive compound. In addition, it is incomplete in that it does not characterize the hepatic effects to make certain that it is comparable to the many studies that have characterized the effects on the liver, but which did not characterize possible reproductive effects. It strongly suggests that further, well-controlled studies be performed.</p> <p><u>Developmental Effects</u></p> <p>The Fawell et al. (1999) and Chernoff et al. (2002) studies contradict each other in several findings.</p> <p><u>Testes</u> p84</p> <p>Li et al. (2011), Chen et al. (2013), Wang, et al. (2012) Li et al. (2008), and Wu et al. (2013) are all out of the same laboratory (X. Han). Ding et al. (2006) has the limitations described above. Liu et al. (2010) also suggests testicular damage, but there are also limitations with this study. Taken together, these studies demonstrate the need for well-controlled studies that specifically address the sub-acute to chronic oral toxicity of</p>	
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	<p>microcystins in the whole animal using the liver, testis, ovaries, kidney and other endpoints suggested in these and other studies.</p> <p><u>7.4 Hazard Characterization</u></p> <p><u>7.4.1 Synthesis and Evaluation of Major Noncancer Effects</u></p> <p><u>7.1.2 Systemic Effects</u></p> <p>Pg 89, As mentioned above:</p> <p>Turner et al., 1990. It should be added that this brief description of two cases notes that the fevers and clinical symptoms in the two army recruits resolved following administration of antibiotics, and serum liver enzyme activity was measured and was normal. Therefore, although this brief case report is used to support adverse pulmonary effects, of microcystins through inhalation, it does not support it and I doubt that the clinical symptoms reported were due to microcystin exposure.</p> <p>Pg42 Falconer et al., 1983. For comparison to Turner et al. this paper by Falconer et al. describes a report of illness in a population who were exposed to a <i>Microcystis aeruginosa</i> bloom via drinking water and suffered liver damage as measured by increases in liver enzyme activity in part of the population. Although not definitive, the symptoms and increases in liver enzyme activities are supported by a large body of experimental studies showing that microcystins cause liver damage, and by the human cases in Brazil in which patients were exposed to microcystin in dialysis water.</p> <p>Pg 90, 3<sup>rd</sup> paragraph, "...Evidence for effects of MC-LR on the male reproductive tract...were reported by Chen et al. (2011) and supported by i.p. data Liu et al. (2010) and Chen et al. (2013)." I would disagree that the effects reported by Chen et al. (2011) are supported by the other studies because of the reasons listed above.</p> <p>Pg 90, 4<sup>th</sup> paragraph, "Effects in the male reproductive system..." the summary is adequate and is an accurate representation of the results of the studies. However, I do not think that they should be considered definitive until they are independently reproduced in other laboratories.</p> <p>Pg 91 2<sup>nd</sup> and 3<sup>rd</sup> paragraphs, These also are accurate summaries of the findings of the studies that are referenced. However, same laboratories, therefore, same limitations.</p> <p>Pg 95, Table 7-13. I am not sure if the Kirpenko et al., 1981 study should appear here since it was not a peer-reviewed publication.</p>	
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<b>Manson</b>	<p>Findings from the epidemiological studies are compelling, and more weight should be given to them, particularly the studies by Falconer et al. (1983) and Liu et al. (2011a). These investigators controlled for the temporal association between algal blooms and changes in liver enzymes (which is not possible in studies of colorectal cancer and hepatocellular carcinoma). The results were consistent across both studies and provide a strong rationale for liver toxicity with human exposure to environmentally relevant levels of microcystins.</p> <p>The acute toxicity studies (oral, inhalation and dermal/ocular exposures) are well-described, as are the short term oral and inhalation studies. None of the subchronic studies reported changes in weight or histopathology of the testes.</p>	
<b>Stump</b>	<p>The authors have done a very thorough job of describing studies that are relevant for hazard identification of microcystins. I agree with the conclusions and believe the critical discussions are accurate based on the available data from the literature.</p>	
<b>Yu</b>	See 2.2 above.	

**2.4 The work of Zhou et al. (2012) and Fardilha et al. (2013) are presented as supportive of a proposed *Mode of Action* for microcystin in its impacts on sperm cells in rats. Do these studies represent a reasonable mode of action for the effects seen in Chen et al. (2011)?**

<b>Reviewer</b>	<b>Comments</b>	<b>Response to Comments</b>
<b>Chou</b>	<p>Study by Zhou et al. (2012) is an in-vitro study of spermatogonia. The results of this study could provide supportive evidence for some of the observed effects on sperm cells if direct impact of MC-LR on spermatogonia is the cause of low sperm counts in vivo at the concerned concentrations. As of now, no study has demonstrated a direct effect of MC-LR on spermatogonia in vivo. The presence of Oatps in rat spermatogonia indicates the uptake of MC-LR is possible, and a direct effect is possible, but they do not provide any evidence for the mode of action in spermatogenesis or the mode of action in testicular toxicity for the low dose in-vivo effects. Many other possible target cells/tissues in the male reproductive system that could be the target of the prevailing and observable effects are yet to be investigated. For example, the decrease in testosterone could be a predominant action of MC-LR in testes. The impact of MC-LR on sperm concentration can be mediated through low testosterone production, but low sperm concentration through the action of Oatps in spermatogonia or PPPs in sperm cells (Fardilha et al., 2013) is unlikely the cause of low testosterone concentrations. The observations by Zhou et al. (2012) and Fardilha et al. (2013) can be used for speculations.</p>	

	Our current understanding of the biochemical nature of microcystins indeed indicates that, in general, Oatps and protein phosphatases are the key players in target cells/organs, and through which many types of testicular cells/tissues can be affected.	
<b>Hooser</b>	<p>The work of Zhou et al. (2012) and Fardilha et al. (2013) are presented as supportive of a proposed <i>Mode of Action</i> for microcystin in its impacts on sperm cells in rats. Do these studies represent a reasonable mode of action for the effects seen in Chen et al. (2011)?</p> <p>These two works are supportive of a proposed Mode of Action for microcystin in its impacts on sperm cells in rats. They are also supportive of a proposed Mode of Action for microcystin in its impacts on sperm cells in mice (Chen et al. (2011)). It should be noted here that the proposed effects on sperm cells in any species come primarily from one laboratory (X. Han) and must be verified in other independent laboratories at other locations.</p>	
<b>Manson</b>	In the description of the Chen et al. (2011) study (p. 69, end of first paragraph), the LOAEL is given as 0.79 mg/kg/day, and it should be 0.79 ug/kg/day. The lack of effect on testes weight in this study is notable given the severe testicular lesions and reduced sperm counts found. Otherwise, the studies by Zhou et al. (2012) and Fardilha et al. (2013) provide highly credible support for the proposed Mode of Action for microcystin.	
<b>Stump</b>	<p>It is clear from the literature that microcystins require facilitated transport by OATp to enter cells. The manuscript from Zhou was able to demonstrate that using standard laboratory methods (isolation of spermatogonia, RT-PCR to measure OATp expression, Immunolabeling and Western blotting for determination of intracellular microcystin). The results clearly show that microcystins enter the spermatogonia and that OATp are present in the testis and spermatogonia. While Zhou did not demonstrate which OATp were responsible for facilitating transport of MC-LR into spermatogonia, previous studies have demonstrated that OATp are responsible for transport of MC-LR into the cell. Therefore, the data from the Zhou manuscript support the mode-of-action that microcystins negatively affect sperm cells in rats.</p> <p>It is also clear from the literature that protein phosphorylation is critical for spermatozoa function. Fardilha was able to demonstrate that numerous protein phosphatases are present in human sperm. These phosphatases have been shown to be important for sperm motility, morphology and fertility. Inhibition of protein phosphatases has been shown to affect sperm motility. Although this manuscript did not investigate</p>	

	microcystins, previous studies have shown that MC-LR can inhibit protein phosphatases. Therefore, the data in this manuscript are supportive of the mode-of-action that microcystins can affect sperm cells through inhibition of protein phosphatases.	
<b>Yu</b>	<p>I do not believe the work of Zhou et al. (2012) and Fardilha et al. (2013) are supportive of a proposed MOA for MC-LR in its impact on sperm cells in rats, and strongly oppose this conclusion. First, despite the adverse effect of MC-LR were observed in the male reproductive system, the target tissue or cells are still unclear. It has never been demonstrated that MC-LR can pass the testis-blood barrier and reach to the seminiferous tubes, to the germ cells including spermatogonia, Sertoli cell or Leydig cells. Although it was listed as one of the goal to measure the MC-LR level in the testis and epididymis by LC-MS in Chen et al., 2011 paper, no result was shown in the paper, and even no discussion of it. Wang et al., 2012 revealed that MC-LR by intraperitoneal injection induced significant decrease in the GnRH expression in a dose- and duration-dependent manner. The serum LH and testosterone exhibited similar trends of change, with both LH and testosterone increased in 30 <math>\mu</math>g/kg b.w./day group after 1 day. And 15 <math>\mu</math>g/kg b.w./day group increased also after 4 days. But after 7 days 30 <math>\mu</math>g/kg b.w./day group fell to control level. While after 14 days, compared to control group, in all concentration-groups both of them decreased significantly. Furthermore, in vitro Leydig cell culture demonstrated that there was no uptake of MC-LR, consistent with the no cytotoxicity of Leydig cells. The results from this study showed that MC-LR affected male mice serum hormones and mRNA GnRH expressions by damaging the hypothalamic-pituitary systems. Second, although various histopathological changes have been reported in the testis, no convincing evidence showing the target cells. The most widely reported changes of the testis were the increase of the empty spaces between the seminiferous tubes. However, as evident from the representative photos from the control animals, there were empty spaces too. The majority of the studies did not use the recommended fixation for the testis because the routine histopathological approach can not preserve the unique structure of the testis. Histopathological evaluation of the testis could provide one of the most sensitive end points for detecting the effects of toxicants. It is routinely applied in the evaluation of male reproductive toxicity. However, "routine" histological such as paraformaldehyde based fixation methods are often inadequate for maintaining the "sensitivity" of this type of evaluation. Improper fixation and inappropriate combinations of fixative and embedding media result in unacceptable histological sections (1). The distortions induced by inadequate methods can make the detection of differences between treated and control tissues nearly impossible at all. As stated in the</p>	

	<p>book chapter 4 by Hess and Moore “Formalin alone should never be used to fix testes to be embedded in paraffin. The best results are obtained in paraffin, using either Bouin's fixative or a primary fixation in neutral buffered formalin (NBF) followed by Bouin's fixative. The benefit of the dual fixation is that the tissues also appear well fixed in GMA medium; therefore, if quantitative data are needed subsequent to a general evaluation of paraffin sections”. (Histological Methods for Evaluation of the Testis, Rex A. Hess and Billy J. Moore in METHODS IN TOXICOLOGY, Volume 3A). In order to assure the result from the experiment with testis, it is highly recommended to apply the guideline developed by the reproductive expert panel, “Recommended Approaches for the Evaluation of Testicular and Epididymal Toxicity” TOXICOLOGIC PATHOLOGY, vol 30, no 4, pp 507–520, 2002. The fixation methods for the testis is widely recommended to use Bouin's-solution in order to preserve the microstructure of testis. Sections are recommended to stain with the Periodic acid Schiff (PAS) technique and count-stained with hematoxylin. The fixation with 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) (pH = 7.4) will generate a lot of artifacts, such as the loosen of the testicular tubes. So far, there is no evidence that treatment of MC-LR target the spermatogonia and lead to the depletion of the spermatogonia in the seminiferous tubes (I have reviewed all the photos of the cross-section of testis published). Liu et al., 2010 reported that lesions such as changes in both spermatogonia and Sertoli cells were seen in animals treated with 12.5 µg MC-LR equivalents/kg. But Liu et al. also claimed that recovery occurred by 48 hours with the tissue resembling the control (Liu et al., 2010). Spermatogonia cells are undifferentiated male germ cell, originating in a seminiferous tubule and dividing into two primary spermatocytes in the production of spermatozoa. Damage or reduction of the pool of spermatogonia cells will result in a decrease of the other type of germ cells. It is very hard to understand that the damage of spermatogonia cells would be recovered within 48 hours. Increased empty spaces or loosened microstructure between the seminiferous tubes suggested that MC-LR might target the Leydig cells, which eventual lead to the decrease of the testosterone level. But the increase of the empty spaces also could be the defects from the testis fixation. Therefore, the application of the in vitro culture of spermatogonia to examine the potential mechanism is questionable. The existence of nontransporting polypeptides (Oatps) in the spermatogonia necessary means that MC-LR could enter into spermatogonia since the in MC-LR has to first pass the blood testis barrier. Also the in vitro observation of uptake of MC-LR by the spermatogonia does not mean uptake in vivo. So far it is very clear that spermatogonia is not the target cells of the MC-LR, therefore, Zhou et al. (2012)'s paper could not provide direct information of the Mode of action for the MC-LR induced adverse effects in the testis.</p>	
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	<p>Fardilha et al. (2013) reported an important research on the protein phosphatases (PPs) of the human sperms, and identified three new serine/threonine-protein PPs, PPP1CB, PPP4C, and PPP6C together with two tyrosine-PPs, MKP1 and PTP1C. It is reasonable to infer from the finding of Fardilha et al. (2013) that inhibition of protein phosphatases can adversely impact sperm motility. But it does not mean that MC-LR can inhibit the activities of these phosphatases. It might be true MC-LR could inhibit those PPs, but in fact, there was no study reporting MC-LR inhibit the human sperm motility through the inhibition of PPs. That “inhibition of protein phosphatases” is the Mode of Action of MC-LR induced-dysfunction of the sperms needs to be further verified. We need to verify the target tissue or target cells of the MC-LR induced male dysfunction. We need to verify whether the decrease of the sperm count or sperm motility is due to the damage of the testis, or due to the damage of testis such as the depletion of spermatogonia in the testis or due to the depletion of the Leydig cells leading to the decrease of the testosterone. We still need to verify whether MC-LR directly inhibits the PPs in the epididymis and impairs the sperm development. We still need to verify whether the MC-LR directly damage the hypothalamic-pituitary systems (Wang et al., 2011), and adverse effect on the sperm count and motility were the secondary effects of the changes of hormones such as FSH, LH and testosterone. Although the paper is very informative and probably imply potential explanation, so far there is no direct evidence supporting that inhibition of PPS is the mechanism of MC-LR induced malformation or decreased count of sperm. Therefore, I do not think Fardilha et al. (2013) studies represent a reasonable mode of action for the effects seen in Chen et al. (2011)?</p>	
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### 3. Chapter 8 - Dose-Response Assessment.

This chapter provides the dose-response assessment and the derivation of RfD.

Reviewer	Comments	Response to Comments
Hooser	<p><b><u>General comments for Chapter 8:</u></b></p> <p><b><u>8.0 Dose-Response Assessment</u></b></p> <p><b><u>8.1 Dose-Response for Noncancer Effects</u></b></p> <p>Human Data</p> <p>Pg 99, 1<sup>st</sup> paragraph, “Human data on...” I think that the link between microcystins and the symptoms reported in Turner et al, 1990, is very weak. Therefore, the symptoms reported in that paper should not be used as a summary for symptoms related to microcystin exposure. It would be better to use the list of symptoms reported in Falconer et al., 1983 or some other report</p>	

	<p>where the exposure to microcystins is clear.</p> <p>Animal Data - Pg 99, 3<sup>rd</sup> paragraph and pg 100, 1<sup>st</sup> paragraph regarding male reproductive toxicity. I agree that these paragraphs accurately summarize data in the studies that are referenced, however, I have the same concerns as listed above and feel that these studies indicate a need for further investigation, but by themselves are not compelling until reproduced in other laboratories at other locations.</p> <p>Table 8-1, Reproductive Toxicity. I would remove the Kirpenko study for the reasons cited above.</p>	
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### 3.1 Data sufficiency

#### 3.1.1 Is the conclusion that there are sufficient data to derive a reference dose (RfD) for microcystin-LR adequately justified? Please discuss and provide suggestions, if needed.

Reviewer	Comments	Response to Comments
<b>Chou</b>	<p>Yes. After Kirpenko et al.'s study is removed and the 40 ug/kg is identified as LOAEL (see comments below, under Section 3.2.4.), the remaining five publications in Table 8-1, collectively, support a range of NOAEL values that is within an order of magnitude.</p> <p><u>The reviewer has additional comments on Chapter 8: p. 99-109:</u></p> <p>p.101, Zhang et al., 2010: Please add decreases in body weight, increase in relative liver weight, and fatty degeneration to the "Responses" column.</p> <p>p. 101, Zhang et al., 2012: See P. 4 of the Supporting Information (Zhang et al., 2012). The effect of 0.2 ug/kg on infiltrating lymphocytes is stated in p. 4 of the Supporting Information and presented in Figures S1B. Please add lymphocyte infiltration to the "Responses" column.</p> <p>p. 101, Kirpenko et al., 1981: Please remove this study from Table 8-1. This study is conducted with an extract from blue-green algae, not MC-LR.</p> <p>p. 103, Paragraph 1 and Paragraph 2, "species of mouse" ("moused", likely a typo in Paragraph 2): Mouse is the species. Perhaps you want to say "strain of mouse".</p> <p>P.103, Paragraph 2, The two sentences in Line 2-4:</p> <p>There are major errors or oversights in these two sentences:</p> <p>(1) Both Chen and the author of this paragraph (Author, hereinafter) directly compare the "ppm" in solution with the "ppm" in air.</p>	

	<p>(2) Both Chen and the Author compared directly toxicity in rats with toxicity in mice.</p> <p>(3) “.. the authors believe that...” Please delete this. An author’s believe is not a scientific justification.</p> <p>(4) Testes weights and body weights should be readily available in a laboratory with quality control. There is no indication that the authors offered to provide the data during the personal communication.</p> <p>(5) It is highly unusual to use epididymis for sperm motility measurement. The normal practice is using mature sperm cells collected from the cauda epididymis. Sperm cells in other parts of the epididymis do not have normal motility. The sperm motility data, therefore, are highly questionable.</p> <p>P.103, Paragraph 3: The data in the study by Chen et al. (2011) cannot be qualified as “the best available data” because of the following reasons. (1) The treatment design is erroneous. (2) The study methods are highly questionable. (3) The quality of data keeping is highly uncertain. (4) Statements in the publication revealed authors’ lack of critical scientific judgment (See comments above and below.). In addition, the dosages in the study by Chen et al. (2011) are calculated without the actual water consumption and body weights. The uncertainties in the data quality are too high for this study to be used as the key study in toxicity assessment.</p>	
<b>Hooser</b>	<p>The conclusion that there are sufficient data to derive a reference dose for MC-LR based on male reproductive effects is not justified because the studies that are described have not been replicated at other independent laboratories at other locations. Therefore, male reproductive effects should not be used as the endpoint. Hepatic effects have been widely shown and established at many different laboratories and should be used as the endpoint until such time as the use of other endpoints has been established.</p>	
<b>Manson</b>	<p>The conclusion that there are sufficient data to derive an RfD for microcystin-LR is well justified. Use of the Chen et al. (2011) study to derive an RfD is valid and supported by the data. Table 8.1 appears to be redundant to Table 7.13 as the same information is provided in both.</p>	
<b>Stump</b>	<p>While there are deficiencies with the reproductive studies that have been performed on microcystins, the authors have identified the deficiencies and I believe have correctly determined that sufficient data is available to derive a reference dose.</p>	

<b>Yu</b>	Due to the concern of the data quality in Chen et al., 2011, as reflected in the incompleteness of the study design, unknown strain of mouse and ages, deficiency in the description of method used in sperm count and sperm motility analysis, inappropriate fixation approach in the histopathological examination of testis, and staining of the cross-section, and descriptive observation of the morphological examination of the histology, it is very hard to justify to use dataset without quality insurance. The dose-response data from Fawell et al., 1999, can be used to derive a RfD for MC-LR.	
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**3.1.2 Have critical data gaps been identified and/or addressed for cyanobacterial toxins? Please discuss and provide suggestions, if needed.**

<b>Reviewer</b>	<b>Comments</b>	<b>Response to Comments</b>
<b>Chou</b>	Chapter 9.0 (p.110) has provided a comprehensive list.	
<b>Hooser</b>	<p>Critical data gaps that need to be addressed:</p> <ol style="list-style-type: none"> <li>1. Male reproductive effects due to sub-acute to chronic, oral administration of MC-LR to male mice/rats need to be replicated at other independent laboratories at other locations and compared to hepatic toxicity in those animals. These studies need to look at all organs and a variety of endpoints in males and females.</li> <li>2. The bioavailability of microcystins in seafood to humans consuming fish and shellfish that have themselves ingested microcystins, needs to be investigated.</li> </ol>	
<b>Manson</b>	<p>I agree that use of methanol as a vehicle for microcystin-LR while water alone was used in the control group is not problematic and should not prevent use of this study for derivation of the RfD. I agree that much more information could have been presented on testes weight, but given the dimension of changes in other sperm parameters, this is not a fatal flaw. May investigators recommend that absolute testes weight be used rather than relative (to body weight) as the two parameters are not linearly related.</p> <p>In the Falwell et al. (1999) 13 week study, all tissues from the control and high dose group were examined histopathologically, and lesions were found in the liver alone. The lack of lesions in the testes at the high dose group is notable and should be included as a critical data gap.</p>	
<b>Stump</b>	The authors have done a good job of identifying the data gaps. I do not have any suggestions for additional data gaps.	

<b>Yu</b>	<p>Please see comments in 2.4.</p> <ol style="list-style-type: none"> <li>1. It is unclear whether the “inhibition of protein phosphatases” is the Mode of Action of MC-LR induced-dysfunction of the sperms.</li> <li>2. There is a need to verify the target tissue or target cells of the MC-LR induced male reproductive dysfunction.</li> <li>3. There is a need to verify whether the decrease of the sperm count or sperm motility is due to the damage of the testis, or due to the damage of testis such as the depletion of spermatogonia in the testis or due to the depletion of the Leydig cells leading to the decrease of the testosterone.</li> <li>4. There is a need to verify whether MC-LR directly inhibits the PPs in the epididymis and impairs the sperm development.</li> <li>5. There is a need to verify whether the MC-LR directly damage the hypothalamic-pituitary systems (Wang et al., 2011), and adverse effect on the sperm count and motility were the secondary effects of the changes of hormones such as FSH, LH and testosterone.</li> </ol>	
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### 3.2 Identification of the critical study.

Please critically review and evaluate the potential key studies Chen et al. (2011) and Fawell et al. (1999) for use in the development of a RfD for microcystin-LR.

Current international guidelines or standards for microcystin-LR in drinking water are based on reported liver effects identified in the subchronic mouse study by Fawell et al. (1999). However, a new study (Chen et al., 2011) assessed reproductive effects in male mice following exposure to microcystin-LR in drinking water and identified sperm count and sperm motility as a sensitive toxicological endpoint.

#### 3.2.1 Are the methodologies of both studies sound? Please discuss the methodologies and their strengths and weaknesses.

<b>Reviewer</b>	<b>Comments</b>	<b>Response to Comments</b>
<b>Chou</b>	<p>There is no study in either publication to evaluate the critical stages of oogenesis, spermatogenesis, fertilization, and embryonic &amp; fetal development.</p> <p>The study by Fawell et al. tested no dose below 40 ug/kg.</p> <p>In the study by Chen et al. (2011), methanol was introduced in the test substance, the dose of methanol increases with the increase of test substance, and there is no appropriate control treatment for the test treatments.</p> <p>In addition, in the study by Chen et al. (2011), sperm</p>	

	concentration and sperm motility were measured in mixed populations of sperm cells. It was a mixture of mature sperm cells from the cauda epididymis and immature sperm cells from the head of epididymis. Furthermore, the culture medium used by Chen et al. does not support the process of sperm capacitation, a process significantly affects sperm motility.	
<b>Hooser</b>	The methodologies of both studies appear to be sound. It is unfortunate that each one of them narrowed the focus of the study to only one system, reproductive or hepatic.	
<b>Manson</b>	See 3.1.1. The Chen et al. (2011) is the most appropriate study to use for the RfD; the lack of testicular findings in the high dose group of the Falwell et al. (1999) is a critical data gap.  See Sections 3.1.1 and 3.1.2 above.	
<b>Stump</b>	<p>The Chen paper used sufficient sample sizes for data interpretation. Deficiencies include:</p> <p>The authors do not present testes weights.</p> <p>MC-LR was dissolved in methanol for the control group water did not contain any methanol.</p> <p>The methodology used for sperm motility is completely lacking.</p> <p>For sperm morphology, the sperm suspension was allowed to dry on the slide which can lead to artifacts. The preferred method is a wet-mount approach.</p> <p>The fixation and staining of testes for microscopic examination were not optimal (Davidson's or Bouin's fixation followed by PAS staining).</p> <p>Otherwise, the data is adequate. The sperm count data shows a clear dose-response at the mid and high doses groups that are more pronounced at 6 months than at 3 months. Similar effects were observed for sperm motility and hormone levels. The TUNEL assay also shows a clear dose-related increase in apoptotic cells at 6 months.</p> <p>With regards to the Fawell manuscript, the 13-week study appears to be a routine toxicology study. Deficiencies include:</p> <p>The methods are very sparse. It is a bit unclear as to what tissues were examined microscopically.</p> <p>No body weight data is presented in the manuscript, only a summary in the text.</p> <p>No data is presented for the developmental toxicity study. Therefore, I have no confidence that the author's</p>	

	<p>conclusions regarding this study are correct.</p> <p>As long as we assume the 13-week study was performed according to standard practices, the conclusions drawn by Fawell (liver microscopic findings were observed in the mid and high dose group), are supported by the data.</p>	
<b>Yu</b>	<p>Regarding the publication of Chen et al., 2011, it is a critical publication regarding the potential effects of microcystin-LR on the male reproductive system. However, the methodology and analysis used in the manuscript lead me to concern about the reliability of the results.</p> <p>The followings are the detailed problems observed in this paper.</p> <p>1. Study design</p> <p>As illustrated in the Figure 1, LC-MS is proposed to measure the concentration of microcystin-LR (MC-LR) in epididymis of 10 mice, and testis of 15 mice, but these data never mentioned in the results. These data will be critical to evaluate the testicular toxicity since it is still unclear whether the MC-LR could pass the Testis-blood barrier, and whether MC-LR distribute to the epididymis.</p> <p>It could be negative or positive results from these LC-MS measurements. However, no mention of LC-MS result in the result section or even no mention in the discussion reflected the quality of the research work. At least, this publication was not a high quality research!</p> <p>Page 552 “ Of 20 mice in each group, the right epididymides from 10 mice were used to carry out the sperm quality and the left 10 epididymides were saved to check the quantitative of MC-LR by LC–MS. Because the volume of serum was limited, all the blood samples in our study were double-diluted. The 10 samples were chosen from 40 samples at random and represented 10 different mice. Five testes from 5 mice were used for histopathological analysis and TUNEL staining. The remaining 15 mice were used for qualitative and quantitative analysis of testicular MC-LR by LC–MS (Fig. 1).”</p> <p>2. Mice, strain and ages</p> <p>There was no information about the strain of the mice. The strain difference in response to chemical treatment is reported to MC-LR. The age of these mice is unclear. Based on the body weight information stated in the paper from 15 to 25 g, and it is assumed the strain of mice is BALB/c, the age of these mice might be between 3 weeks to 8 weeks. There are a huge difference of development of male reproductive system in ages,</p>	

	<p>and response to chemicals is different.</p> <p>3. Sperm Analysis</p> <p>There is a lack of information about the sperm analysis. “It was minced into 1-mm pieces and incubated in 2mL BWW medium at 32 °C for 1 h. Sperm counts were determined through an automatic semen analyzer (VERSION 12.2, HTM-TOX IVOS).”</p> <p>Since the measurement of sperm analysis was carried through the computer-assisted sperm analysis (CASA). There is no description of the analytic protocol. Neither the information about the quantitative parameters of sperm motility obtained from the CASA. The traditional manual examination of sperm count and motility measurement under the microscope is quite subjective; therefore, it is emphasized that the operator should be blinded. However, the importance to declare “This operation was performed by an operator who was blind to the group assignment of animals” is unclear. The lack of description of the analytic protocol as well as the sub-professional statement leads to the concern of the quality of the results.</p> <p>A normal description of CASA from the HTM-IVOS Sperm Analyzer during measurements normally includes the parameters such as minimum contrast, minimum cell size, straightness threshold, path velocity cutoff, progressive minimum path velocity, static head size, static head intensity, and static elongation. The calculation of motility of the sperm is unclear. HTM-TOX IVO (version 12), a computer-assistant sperm analysis (CASA), routinely provides the following information including Total, Static Progressive, Motile, Slow, Bent head, Coiled tail, Distal droplet, and Proximal droplet. Therefore, it can be concluded that the sperm analysis in this paper was not carried out with quality insurance.</p> <p>4. Serum hormone assay</p> <p>As described in the Chen 2011 paper, “the blood samples were taken from the eye”, it is practically impossible to collect 1 ml from the eye. Generally speaking, in Balb mice, the blood accounts 0.04-0.06 ml of BW, or 1.0-1.5 ml blood from a 25 gm mouse. The best yields are obtained if the blood is removed slowly and steadily so that the heart is kept beating as long as possible. There is no description of the protocol for the collection of blood from the eye. Is the mouse under the anesthesia procedure? Therefore, this is another example, raising the concern about the accuracy of the data handling, and quality insurance. As stated in the paper, “Because the volume of serum was limited, all the blood samples in our study were double-diluted”, however, it is unclear how these samples diluted. What is the procedure call “double diluted” ? Is the blood sample or serum diluted? What solution is used to dilate?</p>	
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	<p>The results shown in Figure 3 A and B were mean <math>\pm</math>S.E. The standard deviation for the serum testosterone for the control and the groups without statistical significance are huge, but all the groups with statistic significance were very small. The variation of the testosterone in the control is consistent with the publication, however, the physiological implication of the significant decrease in the S.E. in the high dose groups is unclear, and have not discussed.</p> <p>5. Histopathological evaluation</p> <p>Histopathological evaluation of the testis provides one of the most sensitive end points for detecting the effects of toxicants. It is routinely applied in the evaluation of male reproductive toxicity. However, "routine" histological such as paraformaldehyde based fixation methods are often inadequate for maintaining the "sensitivity" of this type of evaluation. Improper fixation and inappropriate combinations of fixative and embedding media result in unacceptable histological sections (1). The distortions induced by inadequate methods can make the detection of differences between treated and control tissues nearly impossible at all. As stated in the book chapter 4 by Hess and Moore "Formalin alone should never be used to fix testes to be embedded in paraffin. The best results are obtained in paraffin, using either Bouin's fixative or a primary fixation in neutral buffered formalin (NBF) followed by Bouin's fixative. The benefit of the dual fixation is that the tissues also appear well fixed in GMA medium; therefore, if quantitative data are needed subsequent to a general evaluation of paraffin sections" (Histological Methods for Evaluation of the Testis Rex A. Hess and Billy J. Moore in METHODS IN TOXICOLOGY, Volume 3A).</p> <p>It is highly recommended to apply the guideline developed by the reproductive expert panel, "Recommended Approaches for the Evaluation of Testicular and Epididymal Toxicity" TOXICOLOGIC PATHOLOGY, vol 30, no 4, pp 507–520, 2002. The fixation methods for the testis is widely recommended to use Bouin's-solution in order to preserve the microstructure of testis. Sections are recommended to stain with the Periodic acid Schiff (PAS) technique and count-stained with hematoxylin. The fixation with 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) (pH = 7.4) will generate a lot of artifacts, such as the loosen of the testicular tube.</p> <p>Spermatogenesis is a cyclic process during which, within each epithelial area, various generations of germ cells undergo a series of developmental steps according to a fixed time schedule. The cycle of the seminiferous epithelium can be subdivided into stages. In the mouse, 12 such stages have been described that can be distinguished from one another by steps in spermatid development. In order to compare the effect of chemicals on the spermatogenesis, a careful examination of the stage, counting</p>	
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	<p>of different cells in each stage is critical to pinpoint the potential effect on the testis. One example to evaluate the pathological changes in testis can be found in Toxicology and Applied Pharmacology 174, 35–48 (2001). Quantification of the cells in different stage of the tubule is listed.</p> <p>As listed in Figure 4 A, it was claimed that “No significant difference in the spermatogenic epithelium in seminiferous tubules was observed between control and MC-LR treated groups”. Even with a well-experienced histopathological expert, it will be very hard to judge through these representative photos. As stated previously, the paraformaldehyde fixation did not preserve the fine microstructure. Cytoplasmic shrinkage and chromatin aggregations were observed in control and treatment group. Loosen structure leading to numerous empty spaces between cells were observed in all groups.</p> <p>Without quantitative evaluation of the cells in the different stage of the tubules, it is very hard to tell the difference of seminiferous tubes. It is unclear how the author concluded “In comparison with control, the spermatogenic epithelium became sparse at 3.2 □g/L. The structure of the spermatogenic epithelium was at a loss, deranged and thinner at 10□g/L of MC-LR.” It is unclear the authors’ concluded that “the □□□g/L group also showed a loss and derangement of spermatogenic cells, enlargement of the lumen of the seminiferous tubules, thinning of the spermatogenic epithelium (Fig. 4B-d)”. Especially, how the authors concluded that MS-LR treatment lead to “depopulation of Leydig cells, Sertoli cells, and mature sperm” since these pictures did not show clearly where is the Sertoli cells or Leydig cells in the control. Again, these less-professional evaluation of the histopathological changes significantly compromised the quality of the research, and therefore, it need to take serious concern of the result.</p> <p>6. TUNEL Cell counts</p> <p>“The number of the testicular cells in sections which were positive for TUNEL (green) was counted from 10 fields, selected at random and observed under the fluorescence microscope (X400)”. It is very curious how to randomly select 10 fields under microscope. The examiner has to move the stage and observe the microscope field and adjust the focus. This procedure is very subjective and not a random procedure. How the percentage of the TUNEL positive cells are calculated is not described in the paper. Assuming the results in Figure 5 C and 5D are right, then the representative figures in 5A and 5B are misleading. There were no apoptotic cells in Fig 5Aa, b or c. It is also unclear what is the cell type of these apoptotic cells. In summary, due to the lack of detailed information about the protocol used to collect the data, inappropriate methodology used to fix the testis tissue, and the lack of objective and</p>	
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	quantitative evaluation of the morphology, the quality of the research is compromised, and the results are not reliable. Therefore, it needs to take an additional cautious to use these data in the risk assessment.	
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**3.2.2 Are strengths and weaknesses of each study appropriately described? Please provide suggestions, if needed.**

<b>Reviewer</b>	<b>Comments</b>	<b>Response to Comments</b>
<b>Chou</b>	<p>For the study by Chen et al. (2011), the varying amounts of methanol in treatments are not appropriately addressed. The measurements of sperm concentration and sperm motility in mixed populations containing immature sperm cells are not addressed. The type of medium used for sperm motility measurement is not addressed.</p> <p>For the study by Fawell et al. (1999), when the differences in both body weight gains and feed intake are statistically significant, data are not shown. Organ weights, especially liver weights, are not reported. The elevated total incidence of acute and chronic inflammation in the 40 ug/kg treatment, Table 7-7, was not addressed.</p> <p>P. 102, "... the higher doses, the gavage route of administration, and the requirement for tissue uptake via transporters in the Fawell et al. (1999) study introduce uncertainty with regard to the systemic dose.": Please correct this statement. Gavage is an accepted method of oral exposure for oral RfD assessment. Gavage introduces less uncertainty than dosing through ad-lib drinking water in laboratory animal studies. Assessment of RfD is not an assessment for systemic dose.</p>	
<b>Hooser</b>	<p>Many of the strengths and weaknesses of each study are described in the text of the report. I would also add:</p> <ol style="list-style-type: none"> <li>1. Chen et al., 2011 – lack of replication of the male reproductive effects in other independent laboratories in other locations.</li> <li>2. Chen et al., 2011 – Histopathology of testis – the descriptions are inadequate and do not provide sufficient detail to adequately assess the degree of damage.</li> </ol>	
<b>Manson</b>	The strengths and weaknesses are adequately described except for the lack of histopathological effects on the testes at the high dose in the 13 week study by Fawell et al. (1999).	
<b>Stump</b>	The strengths and weaknesses of the Chen paper are assessed very thoroughly by the authors. I would suggest the authors add a couple more points for weaknesses to Section 8.1.1.2. The	

	<p>strain of mouse used in the study was not specified. Chen did not use best practices for microscopic examination of the testis (Davidson's or Bouin's fixation followed by PAS staining). In addition, a functional assessment of fertility (breeding to naive females) would greatly add to the value of the study.</p> <p>The strengths and weaknesses of the Fawell manuscript relative to selection of the key study for RfD determination are adequately described.</p>	
<b>Yu</b>	See comments in 3.2.1.	

**3.2.3 In this document, the Chen et al. (2011) is proposed as the critical study for developing the RfD. Please comment on this selection.**

<b>Reviewer</b>	<b>Comments</b>	<b>Response to Comments</b>
<b>Chou</b>	The erroneous study design precludes the study by Chen et al. (2011) from being considered as a key study for dose-response assessment. See comments on this study through this review.	
<b>Hooser</b>	<p><b><u>8.1.1 RfD Determination</u></b></p> <p><b><u>8.1.1.1 Choice of Key Study</u></b></p> <p>Pg 102. I disagree with the first sentence, "The key study for the development of an RfD for microcystins is that of Chen et al. (2011)..." because the EPA should not base an RfD on studies that have not been replicated at other independent laboratories at other locations regardless of how well those studies were performed at the first laboratory. Therefore, male reproductive effects should not be used as the endpoint. Hepatic effects have been widely shown and established at many different laboratories and should be used as the endpoint until such time as the use of other endpoints have been established.</p>	
<b>Manson</b>	I agree that the Chen et al. (2011) study is the most appropriate based on the route of exposure and quantification of sperm motility and sperm count.	
<b>Stump</b>	I agree with the choice of the Chen study as the critical study because the dose level where effects were observed is much lower than the Fawell studies.	
<b>Yu</b>	<p>See comments in 3.2.1.</p> <p><i>Point of clarification:</i></p> <p>I have clearly given my answers in the review report. The methodology, and description of the protocol and the evaluation of the results were questionable, therefore, it is very hard to</p>	

	defend the data quality. I still believe the data from Fawell et al. 1999 are more defensive than Chen's.	
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**3.2.4 Please comment on the relative merits of Chen et al. (2011) vs Fawell et al. (1999) as the critical study. Which study represents the best available science and most appropriate toxicological endpoint for the basis of an oral RfD for microcystin-LR? Please provide the basis for your conclusion.**

Reviewer	Comments	Response to Comments
<b>Chou</b>	<p>Because of the problems in the study design and methods, and the concerns over the scientific judgments demonstrated in the publication, it is not possible for the reviewer to evaluate its merits with high confidence. See comments through this review.</p> <p>The quality and the design of the study by Fawell et al. (1999) are acceptable.</p> <p>Additional comment: Because of the statistical and biological significance of the decrease in body weight gain and the elevated total incidence of inflammation, 40 ug/kg should be determined to be a LOAEL.</p>	
<b>Hooser</b>	<p>Fawell represents the most appropriate toxicological endpoint and is the better choice because its endpoint and conclusions are supported by numerous studies from different labs around the world. In addition, the NOAEL level found in this study is based on a known concentration of MC-LR that was orally dosed to the animals. Since the adoption of the WHO guideline of 1 ug microcystin /L in drinking water, toxicity at water concentrations of microcystins at or below 1 ug/L have not resulted in human or animal toxicity.</p> <p>The effects on testis and spermatozoa reported in the Chen study have not been replicated and verified by other independent laboratories. Almost all of studies on the reproductive toxicity of MCLR come from this one laboratory and a small group of researchers in China over the past few years. Until these studies are replicated and confirmed in other laboratories, they should not be used to develop an oral RfD for microcystin-LR. In addition, since the water consumption of the mice in the study is not presented, the actual amount of MC-LR ingested by the mice is based on a calculation of the predicted water consumption of the mice in the study.</p>	
<b>Manson</b>	See comments above. The Chen et al. (2011) study has the greatest relative merit and the sperm count and motility data should be used as the basis of an oral RfD.	
<b>Stump</b>	The Chen study is the critical study because it used the most	

	appropriate route of administration (drinking water vs. gavage for the Fawell study) and the dose level where effects were observed (0.79 µg/kg/day vs. 200 µg/kg/day for the Fawell study). While some deficiencies have been identified in the Chen study as addressed in the EPA document and my peer review, these deficiencies are not enough to select the Fawell study as the critical study.	
<b>Yu</b>	See comments in 3.2.1.	

### 3.3 Calculation of RfD.

This Health Effects Support Document proposes an oral RfD for microcystin-LR based on the sperm motility and sperm count effects identified in the Chen et al. (2011).

#### 3.3.1 Is the calculation of the RfD for microcystin-LR clear and accurate? Please discuss and provide suggestions, if needed.

Reviewer	Comments	Response to Comments
<b>Chou</b>	<p><b>3.3</b></p> <p>The study design and measurement methods in the study by Chen et al. (2011) are not acceptable. In addition, sperm counts are a poor measurement for reproductive health or testicular function in human and animals. Healthy and fertile individuals, humans or animals, have a large variation in sperm counts, beyond one standard deviation of the mean.</p> <p>In infertile or sub-fertile individuals, humans or animals, sperm counts are not an acceptable indicator for reproductive function even when low numbers of viable sperms are the true cause of low fertility, because a significant portion of the sperm population in the counts could be non-functional sperms. Sperm counts should never be used as a measurement for general toxicity in RfD assessment.</p> <p>Hypothetically, sperm counts may be used for male reproductive toxicity assessment, but only when (1) all sperm cells in the counts are normal; (2) the test chemical has no other effect that could effect fertility or other reproductive functions; and (3) risk assessors know how to extrapolate the sperm count data from animals to men. Furthermore, using one standard deviation is not an appropriate selection of BMD for sperm counts. In practice, there is currently no acceptable method to derive RfD from sperm counts.</p> <p><b>3.3.1</b></p> <p>No. Please see comments above.</p>	

<b>Hooser</b>	As far as I can assess it, however, this is not my area of specialty.	
<b>Manson</b>	I am not an expert in this area and therefore cannot evaluate the calculation of the RfD. The biological inputs to this model appear accurate to me.	
<b>Stump</b>	The calculation of the RfD is clear and accurate.	
<b>Yu</b>	Yes.	

**3.3.2 Has uncertainty been adequately accounted for in the derivation of the RfD through the use of uncertainty factors? Please discuss and provide suggestions, if needed.**

<b>Reviewer</b>	<b>Comments</b>	<b>Response to Comments</b>
<b>Chou</b>	Please see comments above.	
<b>Hooser</b>	As far as I can assess it, however, this is not my area of specialty.	
<b>Manson</b>	<p>An uncertainty factor of 300 seems excessively high to me. I agree with the 10-fold factor for intraspecies extrapolation and the 3-fold for interspecies variability. The 10-fold factor for database insufficiencies appears arbitrary to me and this factor would more reliably be based on the quality of data available, which is high, rather than on missing data. A 3-fold factor seems more appropriate to me but these are subjective issues.</p> <p><i>Point of Clarification</i></p> <p>The RfD is given in a ug/kg/day unit (0.00008 ug/kg/day). If this value was converted into a ug/L unit, would values be below the limit of detection?</p>	
<b>Stump</b>	I agree with the use of 10x uncertainty factor for the deficiencies in the database. The Chen paper does not even specify the strain of mouse used. Other deficiencies have been noted by the authors and previously in my review. I agree with the interspecies 3x uncertainty factor because of toxicodynamic differences between mice and humans. Finally, I agree with the intraspecies 10x uncertainty factor for potential susceptible individuals in the human population.	
<b>Yu</b>	Yes	

### 3.3.3 Specific Issues to address:

**3.3.3.1-a The control group in Chen et al. (2011) did not receive any methanol to match the amount used to solubilize the microcystin-LR in the treated groups. Would treating the control and experimental group differently with methanol at the levels used in Chen et al. (2011) be anticipated to have an effect on the sperm count and motility? How does the lack of historical control data impact interpretation of the Chen data?**

Reviewer	Comments	Response to Comments
<b>Chou</b>	<p>Intellectually, the curiosity drives the reviewer to entertain this question. The answer is provided in Section 2.3 of this review.</p> <p>In addition to study design and methods, the validity of a study and the uncertainty in the data are evaluated based on the authors' scientific competency. An objective judgment on this study's scientific competency is made by examining the information provided by the authors. The authors have made many erroneous statements. See previous comments about this study, including comments for p.103, Paragraph 2 in Section 3.1.1.1. Here are two additional examples:</p> <p>In the publication by Chen et al. (2011), studies by Solter et al. (2000) and Li et al. (2008) are used to support the statement that "Our recent studies showed that MCs were also toxic to the male reproductive system and in particular the testes were more sensitive than the liver or other organs [13,14]." While the study (liver) by Solter et al. demonstrated a LOAEL of 16 ug/kg i.p. dose for 28 days, the latter study (testis) demonstrated a LOAEL of 5 ug/kg also i.p. dose for 28 days. None of the study examined any dose below each of their respective LOAELs. These two studies do not support Chen et al.'s claim that testis is a more sensitive target organ than liver.</p> <p>In the publication by Chen et al. (2011), study by Backer et al. (2010) was used to support the statement "One study on lakes in Siskiyou County, California revealed a concentration of MCs as high as 10 ug/L in the blooming season." In fact, the article by Backer et al. presented concentrations as high as almost 1000 ug/L in Fig.2a of the publication by Backer et al. (2010), and &gt;1000 ug/L in Fig. 2b.</p> <p><b>How does the lack of historical control data impact interpretation of the Chen data?</b></p> <p>The uncertainty is so high that this study should not be used as the key study.</p>	
<b>Hooser</b>	Following a brief search, I could not find any studies which would indicate that this dosage of methanol would have an	

	adverse reproductive effect. However, in general, I am concerned that the investigators did not use the appropriate negative control which would have been water with the same concentration of methanol in it.	
<b>Manson</b>	The issue of use of methanol as a vehicle in the treated but not the control group has already been addressed. The additional information provided by Chen et al. is highly reassuring that this is not a critical problem.	
<b>Stump</b>	<p>The low levels of methanol that were used to solubilize the MC-LR are not expected to affect sperm count and motility. With regards to sperm count, Chen states that the IVOS was used for the evaluation. This instrument has been used throughout the world for many years to assess sperm count. Sperm counts are very easy to perform. The magnitude of the difference from the control group is large (2- to 3-fold lower in the high dose group) and is dose-related. Therefore, the lack of historical control data is not a major concern for the sperm count data.</p> <p>For the sperm motility data, the lack of information regarding the method used for motility assessment is of concern. I am also concerned about the long incubation period (1 hour). This long incubation period may explain why control motility values are lower than I am used to seeing. It would have been very helpful to see historical control data from the laboratory with regards to normal control motility values. In addition, progressive motility was not assessed. Therefore, I have less confidence in the motility data than in the count data. However, the motility data is very consistent with the sperm count data with regards to magnitude of difference versus control and the dose groups that are affected. While I have less confidence in the motility data, it does help support the use of sperm count as the most sensitive end point for risk assessment.</p>	
<b>Yu</b>	See comments in 3.2.1	

**3.3.3.1-b Chen et al. (2011) did not provide data on testis weights. How does the lack of testis weights impact the interpretation of Chen et al. regarding the significance of the sperm effects? What is the impact on the strength and validity of the study if no information, or incomplete information was provided on how samples were handled and measurements were made (e.g., % sperm motility), mouse species, or the purity of MC-LR?**

Reviewer	Comments	Response to Comments
<b>Chou</b>	The uncertainties of the results of this study are so high that it should not be used as a key study for toxicity assessment. Please also see comments above.	
<b>Hooser</b>	There is no data on testis weights, nor on liver weights or lesions to confirm that the mice were being affected by the MC-LR. The data are incomplete and need to be replicated in other laboratories.	
<b>Manson</b>	Chen et al. (2011) reported that there were no significant differences in testes weight between groups, which is different from saying testes weight data were not collected. I do not consider this to be a fatal flaw given the pronounced effects on sperm count and motility.	
<b>Stump</b>	At 6 months, Chen reports that sperm counts in the 10 µg/L group were more than 3-fold lower than controls. I would be very surprised if sperm count reduced to this extent did not affect testes weight. This assumption is further supported by the testicular histopathology effects in this group. However, the effects on histopathology and sperm count appear to be very strong. Therefore, I do not believe the study can be discounted. The lack of reporting the purity of MC-LR and strain of mouse are also problematic. Therefore, the addition of a 10x uncertainty factor is warranted.	
<b>Yu</b>	Lack of testis weight data, detailed information about the protocol used to collect the sperm count and motility data, inappropriate methodology used to fix the testis tissue, and the lack of objective and quantitative evaluation of the morphology, the quality of the research is severely compromised, and the results are not reliable. The incomplete information just reflected how the samples were handled and measurements were made (e.g., % sperm motility). Therefore, it needs to take an additional cautious to use these data in the risk assessment. <b>Detailed protocol of sperm count See other comments in 3.2.1</b>	

### 3.3.3.2 Are the sperm effects biologically plausible in humans?

Reviewer	Comments	Response to Comments
<b>Chou</b>	Please see comments for Section 3.3.	
<b>Hooser</b>	If humans have testicular OATps which can transport MC-LR into spermatogonia, Sertoli cells and/or Leydig cells, and if these cell types have protein phosphatases which bind to and are inhibited by MCLR, then yes, the sperm effects are biologically plausible in humans.	
<b>Manson</b>	It is highly likely they are biologically plausible for humans, as has been documented for interspecies extrapolation for agents such as cancer chemotherapy agents.	
<b>Stump</b>	Yes.	
<b>Yu</b>	There is no human data on the adverse male reproductive function induced by MC-LR reported so far, even under high exposure levels. It is unclear whether oral exposure to MC-LR could accumulate in the testis or epididymis. At least whether MC-LR inhibit those PPs and impair the human sperm motility is unclear. It is very hard to conclude MC-LR induced effects on animals at low dose are biologically plausible in human.	

### 3.3.3.3 Would the male testicular effects reported by Chen et al. (2011) be anticipated to be reversible?

Reviewer	Comments	Response to Comments
<b>Chou</b>	Do not know. It cannot be determined without valid data.  For intellectual conversations only: it may be.	
<b>Hooser</b>	Difficult to know for certain without further studies in which males were administered a chronic dose of MCLR for a defined number of days and then a group were allowed to recover from the MC-LR exposure. However, if type A spermatogonia are unaffected by MC-LR (which could be determined in a future study), then the effects might be reversible.	
<b>Manson</b>	As has been well documented with dibromochloropropane (DBCP), it is anticipated that the male testicular effects would be reversible upon cessation of treatment. The exact time interval could be calculated if the testicular stage affected were known.	

<b>Stump</b>	This is a difficult question to answer without assessing experimentally. In addition, I am not a pathologist making it difficult for me to draw conclusions on reversibility of the microscopic findings. As long as spermatogonia and Sertoli cells are still present, reversibility is a possibility.	
<b>Yu</b>	Based on the morphological examination of the testis, and also one study in rabbit testis, the MC-LR effect on sperm count or motility is reversible. However, as discussed previously, the target organ, tissue or cells are still unclear so far. If the effect of the MC-LR on the male reproductive system is the secondary effect from the damage of the hypothalamic-pituitary systems (Wang et al., 2011), then it is very hard to tell whether the adverse effects on male reproductive system is reversible or irreversible.	

## General Questions

### 4. Is the document clear and understandable? Please describe and provide suggestions, if needed.

Reviewer	Comments	Response to Comments
<b>Chou</b>	The document is well written, except p. 103-104.	
<b>Hooser</b>	Yes, it is.	
<b>Manson</b>	The document is clear and understandable with the exception of redundancies in Chapters 5 and 7 described above. Overall, it is clear that a lot of hard work went into preparation of the document and it is technically strong.	
<b>Stump</b>	The document is clear, understandable and well written.	
<b>Yu</b>	To some extent. Please refer other comments.	

### 5. Are you aware of any additional data that should be addressed in the document? If so, please provide a reference.

Reviewer	Comments	Response to Comments
<b>Chou</b>	Supporting Information for Zhang (2012) is provided as an email attached file.	
<b>Hooser</b>	Please see comments in previous sections.	

<b>Manson</b>	I have performed independent literature searches and have not found any additional data that would be relevant to include.	
<b>Stump</b>	I am not aware of any additional data that should be addressed.	
<b>Yu</b>	None.	

**6. Are you aware of any additional issues that should be addressed in the document?**

<b>Reviewer</b>	<b>Comments</b>	<b>Response to Comments</b>
<b>Chou</b>	<p>P. vii: Please correct the page numbers in the TABLE OF CONTENTS, from Section 8.1.1.4 to Section 10.0</p> <p>P. 146: The reference “Wu et al., 2014” is cited as “Wu et al., 2013” in the Draft. Its publication date is January 2014, although it was available online in 2013.</p>	
<b>Hooser</b>	<p>a. Abbreviations, pg xi, kg = kilogram.</p> <p>b. Chapter 4, p.19, 2<sup>nd</sup> paragraph, 2<sup>nd</sup> sentence: Should read, “In Lake Ontario, microcystin levels never exceeded 0.008 ug/L in the nearshore and were detected up to 0.076 ug/L in the bays and rivers. However, higher levels of microcystin, up to 1.6 to 10.7 ug/L, were found...” (Marakewicz, 2006).</p> <p>c. Chapter 4, p. 22, 4.3.1 sentence beginning, “However, studies have reported that ingestion of cyanobacterial toxins may induce vomiting...(Puisseux-Dao and Edy, 2006).” This reference is on the use of Medaka fish in environmental toxicology. There are better references for the effects of cyanobacterial toxins on humans.</p> <p>d. The Executive Summary should be updated to reflect any changes.</p>	
<b>Manson</b>	No, the document is extremely thorough and the only issue is that parts of it can be reduced/summarized to make the information more readable.	
<b>Stump</b>	On p.103, there are 2 typographical errors. In the second full sentence “evident” is spelled incorrectly. In the second sentence of the second paragraph, “corresponding” is spelled incorrectly.	
<b>Yu</b>	See all other comments.	



## **Appendix A: Individual Reviewer Comments**



**COMMENTS SUBMITTED BY**

Karen Chou, Ph.D.  
Associate Professor, Department of Animal Science  
Michigan State University  
East Lansing, Michigan



**External Peer Review of the Draft *Health Effects Support Document (HESD)*  
for the Cyanobacterial Toxin Microcystins**

**Responses to Charge Questions from Dr. Karen Chou**

- 1. Chapters 2, 5, and 6** of the HESD provide information on the chemical and physical properties, exposure, and the toxicokinetics of microcystin.

**1.1 Are you aware of any additional data that should be included in the document? If so, please provide.**

None.

**1.2 Is any of the information or conclusions included in the document incorrect, redundant or irrelevant? Please explain.**

**For Chapter 5: p.23-27:**

p. 23, First paragraph, Line 8, “Cyanobacterial cells can bioaccumulate ...”: Should this be “Microcystins can bioaccumulate...”?

p. 23, Paragraph 1, Line 8, “Cyanobacterial cells can bioaccumulate in zooplankton.... and as a result of grazing may settle out of water column leading to an accumulation in the sediment.”: Please use two separate sentences because bioaccumulation and accumulation in the sediment are two different things. Please clarify whether they are the cells or the toxins that are settled out of the water column.

p.23, second paragraph, Line 4-5: This mistake needs to be corrected. The data “3.23 ug/mg dw”, report by Codd et al. (1999), is a microcystin-LR equivalent level of 3 microcystins in the bloom and scum of the irrigation water supply, not a “cyanotoxin level detected in lettuce leaf extracts” as stated in Lines 4-5 of this paragraph.

p. 23, second paragraph, Line 8: Please delete the statement “... of little concern to human health.” It is neither convincing nor informative. Please present concentrations found in plants and estimated level of exposure.

**Chapter 6, p.28-36:**

p. 29, paragraph 4: Adding following information can be helpful in the flow, i.e. building up the knowledge base for the readers:

Microcystins compete with bile acid uptake at a transport system to enter hepatocytes (Thompson and Pace, 1992).

p. 30, paragraph 3, “Covalent adducts of MC-LR, MC-LA, and MC-LL....”: Which study demonstrated this?

p. 30 paragraph 4, (Nisiwaki et al., 1994): Please state the dosages in ug/kg of bw.

p. 30 paragraph 4, Nisiwaki et al., 1994: Please indicate that the i.p. dose is 1000 time higher than the oral dose. (Reviewer's explanation: The dose difference can affect the relative tissue distribution when the tissue or organ uptake depends on saturable or rate limiting transporters.)

p. 30 paragraph 4, Line 4-5 "Small amounts of radiolabel...": Is this truly small amounts or small proportion, % of dose per organ or relative tissue concentrations? Please clarify.

p. 30 paragraph 5, Line 2-3, "The tissue distribution...!": Is this relative amount (% of dose) or absolute concentration? Please clarify.

p. 30 paragraph 5, Line 3-4, "Liver accumulation ...": which dose?

p. 35, fourth paragraph, 4th-3rd to the last line: Is this sentence finished?

p. 36, Third paragraph, (Falconer et al., 1986): What is the species?

**1.3 Please comment on the flow and continuity of these chapters and provide suggestions to enhance the utility of these chapters, if needed.**

These chapters are well written.

**2. Chapter 7 - Hazard Identification.**

This chapter outlines toxicological studies, epidemiology, genotoxicity and mechanistic data. This chapter also includes the characterization of human health effects.

**2.1 Are you aware of any additional critical studies for microcystins that should be included in the document? If so, please provide.**

None

**2.2 Is any of the information included in the document incorrect, redundant or irrelevant? Please describe and provide suggestions, if needed.**

**For Chapter 7, p.37-98:**

p. 37: Paragraph 1, the months of June through September...": What year?

p. 46, Fitzgeorge et al. (1994): Please add the following info for the study by Fitzgeorge et al. (1994): This study used newly weaned CBA/Balbc mice weighing 20 g (+/- 1g). Sex of the mice and the number of mice per group in the tests for LD50 were not reported. Deaths were recorded within 2 hrs of dosing.

p. 46, paragraph 4,: Please specify the age of the "aged" mice.

p. 47, Fitxgeorge et al. (1994): Please consider adding the following information:

This study used newly weaned CBA/Balbc mice, 6 per treatment group, and sex was not specified. Deaths were recorded for 2 hours after a single dose. The estimated LD50 of intranasal instillation, 250 ug/kg, is the same as the LD50 of I.P. exposure, which is much lower than the LD50 of gastric intubation (3000 ug/kg). Aerosol inhalation of 0.005 ug/kg resulted in no death. A single LD50 dose, regardless of the

route of exposure resulted in approximately 45% of liver weight increase. A higher liver weight increases (87%) was observed after the single i.n. dose of 500 ug/kg. While a single i.n. dose of 31.3 ug/kg had no effect on liver weight, repeat doses of i.n. 31.3 ug/kg, once a day for seven days, resulted in a 75% increase in liver weight.

p. 48, Huang et al. (2011), Line 2: Please correct mistake. "Groups of 5 mice...."

p. 52, Fawell et al. (1999): Please indicate that, in addition to "age not specified", body weight is not specified. Reviews comment: This is unfortunate because Body weights and liver weights are important measurements in this study. Initial body weights could have been used to approximate age.

p. 52-53, Fawell et al. (1999), "Mean body weight gain was decreased approximately 15% in all treated male groups." This amount of decrease in mean body weight gain should not be dismissed when considering NOAEL. Liver weight is not reported in the publication.

p. 56, Kirpenlo et al. (1981), "Changes in the estrous cycle...": "Absence of estrus cycle..." is a more specific description. Absence of estrus cycle is reported on p.265 of the cited article.

p. 56, Kirpenlo et al. (1981): Please clarify that the increase in primordial follicles, the decrease in mature follicles, the degeneration of oocytes in Graafian vesicles, the decrease in follicle dimensions, and the increase in the number of involuted corpora lutea were observed after 1.5 months of treatment, while the absence of estrus cycle and atrophy of uterus and genital appendages were observed after 3 months of treatment.

p. 56, Kirpenlo et al. (1981), "Effects on Sertoli cells and spermatogonia were also noted.": Please clarify. "Morphological abnormalities in Sertoli cells and degenerating spermatogonia were also noted."

p. 56, Kirpenlo et al. (1981), A note from the Review: The article is reviewed in details because it is an important study that supports the report by Chen et al. (2011). Unfortunately, the test substance used by Kirpenlo et al. is significantly different from that by Chen et al.

p. 57, Falconer et al. (1988). Please state that the parental mice, 8 females and 2 males, at the age of 20 weeks, received the treatment for 17 weeks before mating.

p. 57, Paragraph 3, Line 6, Liu et al., 2010, "...Sertoli cells, were seen in animals treated with...": Please consider additional info on age. "...Sertoli cells, were seen in immature male Japanese White Rabbits (1.6+/- 0.2 kg) treated with..."

p. 60, Ito et al. (1997b): What is the sex and age of the mice?

p. 62, Paragraph 3, Zhang et al. (2012)"Body weight results were not reported": Body weight results are reported/described in the text of the Supporting Information (p.4), although data are not shown.

p. 62, Paragraph 3, Zhang et al. (2012), "...infiltrating lymphocytes... (doses not specified)." Results of does related infiltrating lymphocytes are provided in Supporting Information in text (p.4) and in Figures S1B and S1C.

p. 63, Please cite reference in the first sentence of Paragraphs 2, 3, and 4.

p. 78, Paragraph 3, “The cell-type specificity of microcystins was investigated using...”: Do you mean “The cell-type specificity of microcystin effects was investigated using...”?

p. 82, Paragraph 2, “Increases in MDA....administered crude extracts...by Li et al. (2011b)”: please indicate routes of exposure.

p. 92, Paragraph 3, “... inhibiting their function (Craig et al., 1996)”, “... inhibiting their functions (Craig et al., 1996)”

p. 97-98, “Potentially Sensitive Populations”: The assertion that “There are gender differences for reproductive effects...” has no supporting data. The i.p. dose of 5 ug MC-LR/kg in mice is an effective dose on serum level of progesterone (Wu et al., 2014), and no dose lower than this has been tested.

**2.3 Are the conclusions and critical discussions for microcystins valid and scientifically defensible? Please describe and provide suggestions, if needed.**

P. 55, Chen et al. (2011): Methanol was present in the treatment doses, but not in the control's. Methanol has known effects on testosterone, FSH and LH (Cooper et al., 1992). Furthermore, synergistic effect of methanol with nutrient and aging factors on testicular function has been demonstrated by (Lee et al., 1991). See references below this paragraph. According to Chen et al. (2011), the MC stock solution was 1 g MC-LR per L of 0.1% methanol solution. The exposure to methanol in the treated animals would be in the same range as the dose of MC-LR, if the reported 0.1% of methanol was a weight to volume expression. On the other hand, the 0.1% is more likely a volume-to-volume expression, therefore, the amount of methanol in the final treatment dosages would be about 80% of the MC-LR doses. These doses are about 1000 times lower than an effect dose of methanol in mice. (The reviewer realized later that this weakness in study design has been discussed on p.102, but believes that it should be stated in this part of the Draft.)

Cooper RL, Mole ML, Rehnberg GL, Goldman JM, McElroy WK, Hein J, et al., 1992. Effect of inhaled methanol on pituitary and testicular hormones in chamber acclimated and non-acclimated rats. *Toxicology* 71(1-2): 69-81.

Lee E, Brady AN, Brabec MJ, Fabel T. 1991. Effects of methanol vapors on testosterone production and testis morphology in rats. *Toxicology and industrial health* 7(4): 261-275.

p. 58, Li et al. (2008): Methanol was present in the treatment doses, but not in the control's. See reviewer's comment above for P. 55, Chen et al. (2011), on methanol. In an effort to examine potential interactions between methanol and MC-LR, the reviewer compared the dose-response relationship of the i.p. route of exposure to MC-LR with methanol (Li et al., 2008) with the same i.p. dosage without methanol (Chen et al., 2013). The former study was conducted in Sprague-Dawley rats of 90-120g for 28 days, and the latter in Wistar rats of 180-200g for 50 days. The decrease in testicular weight was observed in both, although only statistically significant in the latter study. In addition, abnormality of seminiferous tubules was observed in the 5 ug/kg dose group in the former study and it too was observed in the 1 ug/kg group of the latter study. These comparisons indicate that at these dosages there are probably no apparent synergistic effects of methanol and MC-LR on testicular injuries. Such a comparison of the dose-response

relationships of two i.p. studies and the resultant interpretation may alleviate some of the concerns over the presence of methanol in the treatments applied by Chen et al. (2011), but no interpretation can be used to dismiss the fundamental mistake in the study design by Chen et al. (2011). Please note that the authors in Chen et al. (2013) do not overlap with those in Chen et al. (2011) and Li et al. (2008).

**2.4 The work of Zhou et al. (2012) and Fardilha et al. (2013) are presented as supportive of a proposed *Mode of Action* for microcystin in its impacts on sperm cells in rats. Do these studies represent a reasonable mode of action for the effects seen in Chen et al. (2011)?**

Study by Zhou et al. (2012) is an in-vitro study of spermatogonia. The results of this study could provide supportive evidence for some of the observed effects on sperm cells if direct impact of MC-LR on spermatogonia is the cause of low sperm counts in vivo at the concerned concentrations. As of now, no study has demonstrated a direct effect of MC-LR on spermatogonia in vivo. The presence of Oatps in rat spermatogonia indicates the uptake of MC-LR is possible, and a direct effect is possible, but they do not provide any evidence for the mode of action in spermatogenesis or the mode of action in testicular toxicity for the low dose in-vivo effects. Many other possible target cells/tissues in the male reproductive system that could be the target of the prevailing and observable effects are yet to be investigated. For example, the decrease in testosterone could be a predominant action of MC-LR in testes. The impact of MC-LR on sperm concentration can be mediated through low testosterone production, but low sperm concentration through the action of Oatps in spermatogonia or PPPs in sperm cells (Fardiha et al., 2013) is unlikely the cause of low testosterone concentrations. The observations by Zhou et al. (2012) and Fardilha et al. (2013) can be used for speculations. Our current understanding of the biochemical nature of microcystins indeed indicates that, in general, Oatps and protein phosphatases are the key players in target cells/organs, and through which many types of testicular cells/tissues can be affected.

**3. Chapter 8 - Dose-Response Assessment.**

This chapter provides the dose-response assessment and the derivation of RfD.

**3.1 Data sufficiency**

**3.1.1 Is the conclusion that there are sufficient data to derive a reference dose (RfD) for microcystin-LR adequately justified? Please discuss and provide suggestions, if needed.**

Yes. After Kirpenko et al.'s study is removed and the 40 ug/kg is identified as LOAEL (see comments below, under Section 3.2.4.), the remaining five publications in Table 8-1, collectively, support a range of NOAEL values that is within an order of magnitude.

The reviewer has additional comments on Chapter 8: p.99-109:

p. 101, Zhang et al., 2010: Please add decreases in body weight, increase in relative liver weight, and fatty degeneration to the "Responses" column.

p. 101, Zhang et al., 2012: See P. 4 of the Supporting Information (Zhang et al., 2012). The effect of 0.2 ug/kg on infiltrating lymphocytes is stated in p. 4 of the Supporting Information and presented in Figures S1B. Please add lymphocyte infiltration to the "Responses" column.

p. 101, Kirpenko et al., 1981: Please remove this study from Table 8-1. This study is conducted with an extract from blue-green algae, not MC-LR.

p. 103, Paragraph 1 and Paragraph 2, “species of mouse” (“moused”, likely a typo in Paragraph 2): Mouse is the species. Perhaps you want to say “strain of mouse”.

p.103, Paragraph 2, The two sentences in Line 2-4:

There are major errors or oversights in these two sentences:

- (1) Both Chen and the author of this paragraph (Author, hereinafter) directly compare the “ppm” in solution with the “ppm” in air.
- (2) Both Chen and the Author compared directly toxicity in rats with toxicity in mice.
- (3) “... the authors believe that...” Please delete this. An author’s believe is not a scientific justification.
- (4) Testes weights and body weights should be readily available in a laboratory with quality control. There is no indication that the authors offered to provide the data during the personal communication.
- (5) It is highly unusual to use epididymis for sperm motility measurement. The normal practice is using mature sperm cells collected from the cauda epididymis. Sperm cells in other parts of the epididymis do not have normal motility. The sperm motility data, therefore, are highly questionable.

P.103, Paragraph 3: The data in the study by Chen et al. (2011) cannot be qualified as “the best available data” because of the following reasons. (1) The treatment design is erroneous. (2) The study methods are highly questionable. (3) The quality of data keeping is highly uncertain. (4) Statements in the publication revealed authors’ lack of critical scientific judgment (See comments above and below.). In addition, the dosages in the study by Chen et al. (2011) are calculated without the actual water consumption and body weights. The uncertainties in the data quality are too high for this study to be used as the key study in toxicity assessment.

**3.1.2 Have critical data gaps been identified and/or addressed for cyanobacterial toxins? Please discuss and provide suggestions, if needed.**

Chapter 9.0 (p.110) has provided a comprehensive list.

**3.2 Identification of the critical study.**

Please critically review and evaluate the potential key studies Chen et al. (2011) and Fawell et al. (1999) for use in the development of a RfD for microcystin-LR.

Current international guidelines or standards for microcystin-LR in drinking water are based on reported liver effects identified in the subchronic mouse study by Fawell et al. (1999). However, a new study (Chen et al., 2011) assessed reproductive effects in male mice following exposure to microcystin-LR in drinking water and identified sperm count and sperm motility as a sensitive toxicological endpoint.

**3.2.1 Are the methodologies of both studies sound? Please discuss the methodologies and their strengths and weaknesses.**

There is no study in either publication to evaluate the critical stages of oogenesis, spermatogenesis, fertilization, and embryonic & fetal development.

The study by Fawell et al. tested no dose below 40 ug/kg.

In the study by Chen et al. (2011), methanol was introduced in the test substance, the dose of methanol increases with the increase of test substance, and there is no appropriate control treatment for the test treatments.

In addition, in the study by Chen et al. (2011), sperm concentration and sperm motility were measured in mixed populations of sperm cells. It was a mixture of mature sperm cells from the cauda epididymis and immature sperm cells from the head of epididymis. Furthermore, the culture medium used by Chen et al. does not support the process of sperm capacitation, a process significantly affects sperm motility.

**3.2.2 Are strengths and weaknesses of each study appropriately described? Please provide suggestions, if needed.**

For the study by Chen et al. (2011), the varying amounts of methanol in treatments are not appropriately addressed. The measurements of sperm concentration and sperm motility in mixed populations containing immature sperm cells are not addressed. The type of medium used for sperm motility measurement is not addressed.

For the study by Fawell et al. (1999), when the differences in both body weight gains and feed intake are statistically significant, data are not shown. Organ weights, especially liver weights, are not reported. The elevated total incidence of acute and chronic inflammation in the 40 ug/kg treatment, Table 7-7, was not addressed.

P. 102, "... the higher doses, the gavage route of administration, and the requirement for tissue uptake via transporters in the Fawell et al. (1999) study introduce uncertainty with regard to the systemic dose.": Please correct this statement. Gavage is an accepted method of oral exposure for oral RfD assessment. Gavage introduces less uncertainty than dosing through ad-lib drinking water in laboratory animal studies. Assessment of RfD is not an assessment for systemic dose.

**3.2.3 In this document, the Chen et al. (2011) is proposed as the critical study for developing the RfD. Please comment on this selection.**

The erroneous study design precludes the study by Chen et al. (2011) from being considered as a key study for dose-response assessment. See comments on this study through this review.

**3.2.4 Please comment on the relative merits of Chen et al. (2011) vs Fawell et al. (1999) as the critical study. Which study represents the best available science and most appropriate toxicological endpoint for the basis of an oral RfD for microcystin-LR? Please provide the basis for your conclusion.**

Because of the problems in the study design and methods, and the concerns over the scientific judgments demonstrated in the publication, it is not possible for the reviewer to evaluate its merits with high confidence. See comments through this review.

The quality and the design of the study by Fawell et al. (1999) are acceptable.

Additional comment: Because of the statistical and biological significance of the decrease in body weight gain and the elevated total incidence of inflammation, 40 ug/kg should be determined to be a LOAEL.

**3.3 Calculation of RfD.**

This Health Effects Support Document proposes an oral RfD for microcystin-LR based on the sperm motility and sperm count effects identified in the Chen et al. (2011).

The study design and measurement methods in the study by Chen et al. (2011) are not acceptable. In addition, sperm counts are a poor measurement for reproductive health or testicular function in human and animals. Healthy and fertile individuals, humans or animals, have a large variation in sperm counts, beyond one standard deviation of the mean.

In infertile or sub-fertile individuals, humans or animals, sperm counts are not an acceptable indicator for reproductive function even when low numbers of viable sperms are the true cause of low fertility, because a significant portion of the sperm population in the counts could be non-functional sperms. Sperm counts should never be used as a measurement for general toxicity in RfD assessment.

Hypothetically, sperm counts may be used for male reproductive toxicity assessment, but only when (1) all sperm cells in the counts are normal; (2) the test chemical has no other effect that could effect fertility or other reproductive functions; and (3) risk assessors know how to extrapolate the sperm count data from animals to men. Furthermore, using one standard deviation is not an appropriate selection of BMD for sperm counts. In practice, there is currently no acceptable method to derive RfD from sperm counts.

**3.3.1 Is the calculation of the RfD for microcystin-LR clear and accurate? Please discuss and provide suggestions, if needed.**

No. Please see comments above.

**3.3.2 Has uncertainty been adequately accounted for in the derivation of the RfD through the use of uncertainty factors? Please discuss and provide suggestions, if needed.**

Please see comments above.

### 3.3.3 Specific Issues to address:

#### 3.3.3.1-a **The control group in Chen et al. (2011) did not receive any methanol to match the amount used to solubilize the microcystin-LR in the treated groups. Would treating the control and experimental group differently with methanol at the levels used in Chen et al. (2011) be anticipated to have an effect on the sperm count and motility?**

Intellectually, the curiosity drives the reviewer to entertain this question. The answer is provided in Section 2.3 of this review.

In addition to study design and methods, the validity of a study and the uncertainty in the data are evaluated based on the authors' scientific competency. An objective judgment on this study's scientific competency is made by examining the information provided by the authors. The authors have made many erroneous statements. See previous comments about this study, including comments for p.103, Paragraph 2 in Section 3.1.1.1. Here are two additional examples:

In the publication by Chen et al. (2011), studies by Solter et al. (2000) and Li et al. (2008) are used to support the statement that "Our recent studies showed that MCs were also toxic to the male reproductive system and in particular the testes were more sensitive than the liver or other organs [13,14]." While the study (liver) by Solter et al. demonstrated a LOAEL of 16 ug/kg i.p. dose for 28 days, the latter study (testis) demonstrated a LOAEL of 5 ug/kg also i.p. dose for 28 days. None of the study examined any dose below each of their respective LOAELs. These two studies do not support Chen et al.'s claim that testis is a more sensitive target organ than liver.

In the publication by Chen et al. (2011), study by Backer et al. (2010) was used to support the statement "One study on lakes in Siskiyou County, California revealed a concentration of MCs as high as 10 ug/L in the blooming season." In fact, the article by Backer et al. presented concentrations as high as almost 1000 ug/L in Fig.2a of the publication by Backer et al. (2010), and >1000 ug/L in Fig. 2b.

#### **How does the lack of historical control data impact interpretation of the Chen data?**

The uncertainty is so high that this study should not be used as the key study.

#### 3.3.3.1-b **Chen et al. (2011) did not provide data on testis weights. How does the lack of testis weights impact the interpretation of Chen et al. regarding the significance of the sperm effects? What is the impact on the strength and validity of the study if no information, or incomplete information was provided on how samples were handled and measurements were made (e.g., % sperm motility), mouse species, or the purity of MC-LR?**

The uncertainties of the results of this study are so high that it should not be used as a key study for toxicity assessment. Please also see comments above.

#### 3.3.3.2 **Are the sperm effects biologically plausible in humans?**

Please see comments for Section 3.3.

**3.3.3.3 Would the male testicular effects reported by Chen et al. (2011) be anticipated to be reversible?**

Do not know. It cannot be determined without valid data.

For intellectual conversations only: it may be.

**General Questions**

**4. Is the document clear and understandable? Please describe and provide suggestions, if needed.**

The document is well written, except p. 103-104.

**5. Are you aware of any additional data that should be addressed in the document? If so, please provide a reference.**

Supporting Information for Zhang (2012) is provided as an email attached file.

**6. Are you aware of any additional issues that should be addressed in the document?**

P. vii: Please correct the page numbers in the TABLE OF CONTENTS, from Section 8.1.1.4 to Section 10.0.

P. 146: The reference “Wu et al., 2014” is cited as “Wu et al., 2013” in the Draft. Its publication date is January 2014, although it was available online in 2013.

**COMMENTS SUBMITTED BY**

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**External Peer Review of the Draft *Health Effects Support Document (HESD)*  
for the *Cyanobacterial Toxin Microcystins***

**Responses to Charge Questions from Dr. Stephen B. Hooser**

- 1. Chapters 2, 5, and 6** of the HESD provide information on the chemical and physical properties, exposure, and the toxicokinetics of microcystin.

**1.1 Are you aware of any additional data that should be included in the document? If so, please provide.**

a. 5.1.pg 23. Exposures from soil and edible plants: Data gap – Are microcystins bound in plants following uptake by the plant? After ingestion by mammals, are they available for binding tissues followed by toxicity in the person eating the plant?

b. 5.2.pg.23. Exposures from fish and shellfish consumption: Data gap – same question as “a”, except are microcystins bound in seafood tissues following ingestion by the fish, shellfish, etc?”

Q: Are there any documented cases microcystin toxicity in people or animals following ingestion of fish or shellfish that have ingested/been exposed to microcystins? Following ingestion of microcystins by fish or shellfish, the microcystins may be covalently bound to fish/shellfish protein and unavailable to cause toxicity in the people or animals eating them.

Williams DE, Dawe SC, Kent ML, Andersen RJ, Craig M, Holmes CF. [Bioaccumulation and clearance of microcystins from salt water mussels, \*Mytilus edulis\*, and in vivo evidence for covalently bound microcystins in mussel tissues.](#) *Toxicon*. 1997 Nov;35(11):1617-25.

Ibelings, BW, et al. Distribution of microcystins in a lake foodweb. No evidence for biomagnification. *Microbial Ecology* 49, 487-500, 2005. Website for Ibelings: <http://www.unige.ch/forel/Ecologie-microbienne/Equipe/IbelingsB.html>

Dionisio Piers, L.M. Assimilation and depuration of microcystin-LR by the zebra mussel, *Dreissena polymorpha*. *Aquatic Toxicol.*, 69, 385-396, 2004.

Dionisio Pires, L.M. ; Ibelings, B.W. ; Donk, E. van. Zebra mussels as a potential tool in the restoration of eutrophic shallow lakes, dominated by toxic cyanobacteria. In: Velde, G. Van der ; Rajagopal, S.; Vaate, A.A. Bij de (ed.), *The Zebra Mussel in Europe*, pp. 361-372, 2009. Leiden: Backhuys Publishers.

John Fournie, Elizabeth Hilborn, Geoffrey Codd, Michael Coveney, Juli Dyble, Karl Havens, Bas Ibelings, Jan Landsberg, Wayne Litaker. Environmental Protection Agency Papers, Paper 37, [Cyanobacterial Harmful Algal Blooms: Chapter 31: Ecosystem Effects Workgroup Report](#), 2008.

Identifies some data gaps related to cyanobacterial toxins in the aquatic environment. It does not address the issue of bioavailability of microcystins to humans, mammals and birds eating fish and shellfish following uptake by those aquatic organisms.

Data gap - 6.2 Distribution p29: Could not find a radiolabel study which described distribution of radiolabeled microcystin to all tissues accounting for 100% of label. In particular for this Health Effects

Document, distribution to testis was not found in references. Most radiolabel (or immunohistochemical) studies that I had time to look up appear to leave out testis and ovaries.

Q: Or, is it that testis or ovary were examined, but did not have any radioactivity or staining?

6.2. Distribution, Oral, p31, 1<sup>st</sup> paragraph – MC-LR was not found in milk of dairy cattle that were exposed to *M. aeruginosa* cells via drinking water...MC-LR was not found in muscle of beef cattle fed *M. aeruginosa* cells either

Orr PT, Jones GJ, Hunter RA, Berger K. Exposure of beef cattle to sub-clinical doses of *Microcystis aeruginosa*: toxin bioaccumulation, physiological effects and human health risk assessment. *Toxicon* 41, 613-620, 2003.

Correction – p32, under, “Liver Tissues – *in vitro*”, 1<sup>st</sup> paragraph: The statement, “A study done in 1998 showed adverse effects in liver caused by MC (Theiss et al., 1988). As a result, many researchers have examined the distribution to the liver using cell cultures.”, is inaccurate. Well before this study, it was already well established that the liver was the major target organ for microcystin toxicity. Therefore, since in field and experimental instances of microcystin toxicity it was observed and established that the liver was the primary target organ, many researchers have examined the distribution to the liver using perfused liver and hepatic cell cultures.

Clarification, p33, “Liver Tissues – *in vitro*”, 3<sup>rd</sup> paragraph, “Chong et al. (2000) evaluated microcystin toxicity in eight permanent cell lines..., only two of which showed cytotoxicity following MC-LR exposure.” This is to be expected as the preceding paragraph explains that primary cultures of liver cells cease to express OATps after being maintained in culture. If cultured cells of any type don’t have OATps, then the amount of microcystin that makes it into the cells will be very small.

6.3 Metabolism – p34, 3<sup>rd</sup> paragraph: Clarification - *Microcystis* toxin 7820 here and elsewhere refers to microcystin produced by *Microcystis aeruginosa* strain 7820. Strain 7820 primarily produced MC-LR. I do not recall if it produced any other microcystin congeners. In the 1980s, *Microcystis aeruginosa* strain 7820 was being cultured by Dr. W. Carmichael who provided the toxin to other researchers.

**1.2 Is any of the information, or are any of the conclusions included in the document incorrect, redundant or irrelevant? Please explain.**

5.1 and 5.2 p23-24 – The health risk to humans and animals by consumption of fish and shellfish depends not only on the bioaccumulation of toxins in edible fish tissue, but also the bioavailability of active toxin that is present and has sufficient activity to cause toxicity in the humans and animals.

**1.3 Please comment on the flow and continuity of these chapters and provide suggestions to enhance the utility of these chapters, if needed.**

Flow and continuity good.

## 2. Chapter 7 - Hazard Identification.

This chapter outlines toxicological studies, epidemiology, genotoxicity and mechanistic data. This chapter also includes the characterization of human health effects.

### 2.1 Are you aware of any additional critical studies for microcystins that should be included in the document? If so, please provide.

Yes, there is no mention of the very critical studies describing case reports and analyses of serum and tissues from fatal and non-fatal human dialysis patients with liver damage that were exposed to microcystins in dialysis water that was obtained from surface water sources in Brazil. Although the patients in these cases had pre-existing disease, and exposure is intravenous rather than oral, the exposure to microcystins from surface water sources, presence of microcystins in serum and liver, and subsequent liver damage is clear and demonstrates the systemic effects of microcystin in humans. Many animal and *in vitro* studies, verified in many different laboratories support the distribution and uptake by the liver with subsequent hepatic damage which can be severe and fatal. These reports should be summarized in 7.1.2 Systemic Effects, or in a section of their own.

A partial list of references to include and summarize:

1. Carmichael WW, Azevedo SM, An JS, Molica RJ, Jochimsen EM, Lau S, Rinehart KL, Shaw GR, Eaglesham GK. Human fatalities from cyanobacteria: chemical and biological evidence for cyanotoxins. *Environ Health Perspect.* 2001 Jul;109(7):663-8.
2. Jochimsen EM, Carmichael WW, An JS, Cardo DM, Cookson ST, Holmes CE, Antunes MB, de Melo Filho DA, Lyra TM, Barreto VS, Azevedo SM, Jarvis WR. Liver failure and death after exposure to microcystins at a hemodialysis center in Brazil. *N Engl J Med.* 1998 Mar 26;338(13):873-8.
3. Hilborn ED, Soares RM, Servaites JC, Delgado AG, Magalhães VF, Carmichael WW, Azevedo SM. Sublethal microcystin exposure and biochemical outcomes among hemodialysis patients. *PLoS One.* 2013 Jul 24;8(7):e69518. doi: 10.1371/journal.pone.0069518. Print 2013.
4. Hilborn ED, Carmichael WW, Soares RM, Yuan M, Servaites JC, Barton HA, Azevedo SM. Serologic evaluation of human microcystin exposure. *Environ Toxicol.* 2007 Oct;22(5):459-63.
5. Soares RM, Yuan M, Servaites JC, Delgado A, Magalhães VF, Hilborn ED, Carmichael WW, Azevedo SM. Sublethal exposure from microcystins to renal insufficiency patients in Rio de Janeiro, Brazil. *Environ Toxicol.* 2006 Apr;21(2):95-103.
6. Hilborn ED, Carmichael WW, Yuan M, Azevedo SM. A simple colorimetric method to detect biological evidence of human exposure to microcystins. *Toxicon.* 2005 Aug;46(2):218-21.
7. Azevedo SM, Carmichael WW, Jochimsen EM, Rinehart KL, Lau S, Shaw GR, Eaglesham GK. Human intoxication by microcystins during renal dialysis treatment in Caruaru-Brazil. *Toxicology.* 2002 Dec 27;181-182:441-6.
8. Pouria S, et al., Fatal microcystin intoxication in haemodialysis unit in Cararu, Brazil. *Lancet* 352, 21-26, 1998.

**2.2 Is any of the information included in the document incorrect, redundant or irrelevant? Please describe and provide suggestions, if needed.**

**7.1 Human Effects**

**7.1.1 Epidemiological Studies p37**

The Executive Summary on p2 adequately summarizes the limitations of the epidemiological studies that are presented in this section.

Zhou et al., 2002: Table 7-1, p38, Relative Risk of Colorectal Cancer and Microcystin Concentration by Drinking Water Source.

As explained in the paragraph below it beginning, “This study provides suggestive...,” the title of this table is misleading and because of its prominent placing should be changed to, “Relative Risk of Colorectal Cancer by Drinking Water Source.”

Figure 7-1, p39. Similarly, the title of this figure is also misleading because it gives the impression that there is a definitive relationship between colorectal cancer and microcystin exposure when the summary on p38 explains that the study may not have been adequately controlled.

**7.1.2 Systemic Effects**

Pg41 Turner et al., 1990. It should be added that this brief description of two cases notes that the fevers and clinical symptoms in the two army recruits resolved following administration of antibiotics, and serum liver enzyme activity was measured and was normal. Therefore, although this brief case report is used to support adverse pulmonary effects, of microcystins through inhalation, it does not support it and I doubt that the clinical symptoms reported were due to microcystin exposure.

Pg42 Falconer et al., 1983. For comparison to Turner et al. this paper by Falconer, et al. describes a report of illness in a population who were exposed to a *Microcystis aeruginosa* bloom via drinking water and suffered liver damage as measured by increases in liver enzyme activity in part of the population. Although not definitive, the symptoms and increases in liver enzyme activities are supported by a large body of experimental studies showing that microcystins cause liver damage, and by the human cases in Brazil in which patients were exposed to microcystin in dialysis water.

Omission of important studies - There is no mention of the very important studies describing case reports and analyses of serum and tissues from fatal and non-fatal human dialysis patients with liver damage that were exposed to microcystins in dialysis water that was obtained from surface water sources in Brazil. Although the patients in these cases had pre-existing disease, and exposure is intravenous rather than oral, the exposure to microcystins from surface water sources, presence of microcystins in serum and liver, and subsequent liver damage is clear and demonstrates the systemic effects of microcystin in humans. Many animal and *in vitro* studies, verified in many different laboratories support the distribution and uptake by the liver with subsequent hepatic damage which can be severe and fatal. These reports should be summarized in 7.1.2 Systemic Effects, or in a section of their own.

**2.3 Are the conclusions and critical discussions for microcystins valid and scientifically defensible?  
Please describe and provide suggestions, if needed.**

7.2.5 Developmental/Reproductive Toxicity

Reproductive Effects

Oral

Chen et al., 2011, p55. Paragraph beginning, “Sperm quality...” It should be made clear that the manuscript by Chen et al., 2011 did not include the calculations for estimation of oral dose in the mice in this study. These calculations were made after the fact, presumably by those preparing this summary. It would be more appropriate to move these calculations to section 8.1.1, RfD Determination.

Kirpenko et al., 1981, pg 56 – This reference is from a non-peer reviewed study published as a book chapter. I could not find an associated peer-reviewed, published manuscript. This study was performed with a natural population of what is reported to be *M. aeruginosa* at a time prior to purification, identification and chemical characterization of MC-LR. Therefore, while interesting and worthy of follow-up studies, this study itself does not provide support for reproductive or developmental effects of microcystin.

Falconer, 1988 – This reference should be Falconer, et al. (1988). While this reference is from a peer-reviewed manuscript, it too utilized a bloom of what is reported to be *M. aeruginosa* at a time prior to purification, identification and chemical characterization of MC-LR. For the most part, it contradicts the studies of Kirpenko, 1981 and Chen, 2011. Therefore, it indicates a need for further reproductive and developmental studies.

Other Routes

Li et al. (2011), Chen et al. (2013), Li et al. (2008), and Wu et al. (2013) are all out of the same laboratory (X. Han). The only study from another laboratory is the study by Ding et al. (2006) (p58). This study used a crude extract from a *Microcystis sp.* bloom which is shown to contain microcystin LR as one of its components. This study is suggestive of a reproductive effect of *Microcystis sp.* bloom material, but does not specifically identify microcystin as the reproductive compound. In addition, it is incomplete in that it does not characterize the hepatic effects to make certain that it is comparable to the many studies that have characterized the effects on the liver, but which did not characterize possible reproductive effects. It strongly suggests that further, well-controlled studies be performed.

Developmental Effects

The Fawell et al. (1999) and Chernoff et al. (2002) studies contradict each other in several findings.

Testes p84

Li et al. (2011), Chen et al. (2013), Wang et al. (2012) Li et al. (2008), and Wu et al. (2013) are all out of the same laboratory (X. Han). Ding et al. (2006) has the limitations described above. Liu et al. (2010) also suggests testicular damage, but there are also limitations with this study. Taken together, these studies demonstrate the need for well-controlled studies that specifically address the sub-acute to chronic oral

toxicity of microcystins in the whole animal using the liver, testis, ovaries, kidney and other endpoints suggested in these and other studies.

#### **7.4 Hazard Characterization**

##### **7.4.1 Synthesis and Evaluation of Major Noncancer Effects**

##### **7.1.2 Systemic Effects**

Pg 89, As mentioned above:

Turner et al., 1990. It should be added that this brief description of two cases notes that the fevers and clinical symptoms in the two army recruits resolved following administration of antibiotics, and serum liver enzyme activity was measured and was normal. Therefore, although this brief case report is used to support adverse pulmonary effects, of microcystins through inhalation, it does not support it and I doubt that the clinical symptoms reported were due to microcystin exposure.

Pg42 Falconer et al., 1983. For comparison to Turner et al. this paper by Falconer, et al. describes a report of illness in a population who were exposed to a *Microcystis aeruginosa* bloom via drinking water and suffered liver damage as measured by increases in liver enzyme activity in part of the population. Although not definitive, the symptoms and increases in liver enzyme activities are supported by a large body of experimental studies showing that microcystins cause liver damage, and by the human cases in Brazil in which patients were exposed to microcystin in dialysis water.

Pg 90, 3<sup>rd</sup> paragraph, "...Evidence for effects of MC-LR on the male reproductive tract...were reported by Chen et al. (2011) and supported by i.p. data Liu et al. (2010) and Chen et al. (2013)." I would disagree that the effects reported by Chen et al. (2011) are supported by the other studies because of the reasons listed above.

Pg 90, 4<sup>th</sup> paragraph, "Effects in the male reproductive system..." the summary is adequate and is an accurate representation of the results of the studies. However, I do not think that they should be considered definitive until they are independently reproduced in other laboratories.

Pg 91 2<sup>nd</sup> and 3<sup>rd</sup> paragraphs, These also are accurate summaries of the findings of the studies that are referenced. However, same laboratories, therefore, same limitations.

Pg 95, Table 7-13. I am not sure if the Kirpenko et al., 1981 study should appear here since it was not a peer-reviewed publication.

#### **2.4 The work of Zhou et al. (2012) and Fardilha et al. (2013) are presented as supportive of a proposed *Mode of Action* for microcystin in its impacts on sperm cells in rats. Do these studies represent a reasonable mode of action for the effects seen in Chen et al. (2011)?**

These two works are supportive of a proposed Mode of Action for microcystin in its impacts on sperm cells in rats. They are also supportive of a proposed Mode of Action for microcystin in its impacts on sperm cells in mice (Chen et al. (2011)). It should be noted here that the proposed effects on sperm cells

in any species come primarily from one laboratory (X. Han) and must be verified in other independent laboratories at other locations.

### **3. Chapter 8 - Dose-Response Assessment.**

This chapter provides the dose-response assessment and the derivation of RfD.

#### **General comments for Chapter 8:**

#### **8.0 Dose-Response Assessment**

#### **8.1 Dose-Response for Noncancer Effects**

Human Data - Pg 99, 1<sup>st</sup> paragraph, “Human data on...” I think that the link between microcystins and the symptoms reported in Turner et al., 1990, is very weak. Therefore, the symptoms reported in that paper should not be used as a summary for symptoms related to microcystin exposure. It would be better to use the list of symptoms reported in Falconer et al., 1983 or some other report where the exposure to microcystins is clear.

Animal Data - Pg 99, 3<sup>rd</sup> paragraph and pg 100, 1<sup>st</sup> paragraph regarding male reproductive toxicity. I agree that these paragraphs accurately summarize data in the studies that are referenced, however, I have the same concerns as listed above and feel that these studies indicate a need for further investigation, but by themselves are not compelling until reproduced in other laboratories at other locations.

Table 8-1, Reproductive Toxicity. I would remove the Kirpenko study for the reasons cited above.

#### **3.1 Data sufficiency**

##### **3.1.1 Is the conclusion that there are sufficient data to derive a reference dose (RfD) for microcystin-LR adequately justified? Please discuss and provide suggestions, if needed.**

The conclusion that there are sufficient data to derive a reference dose for MC-LR based on male reproductive effects is not justified because the studies that are described have not been replicated at other independent laboratories at other locations. Therefore, male reproductive effects should not be used as the endpoint. Hepatic effects have been widely shown and established at many different laboratories and should be used as the endpoint until such time as the use of other endpoints has been established.

##### **3.1.2 Have critical data gaps been identified and/or addressed for cyanobacterial toxins? Please discuss and provide suggestions, if needed.**

Critical data gaps that need to be addressed;

1. Male reproductive effects due to sub-acute to chronic, oral administration of MC-LR to male mice/rats need to be replicated at other independent laboratories at other locations and compared to hepatic toxicity in those animals. These studies need to look at all organs and a variety of endpoints in males and females.
2. The bioavailability of microcystins in seafood to humans consuming fish and shellfish that have themselves ingested microcystins, needs to be investigated.

### **3.2 Identification of the critical study.**

Please critically review and evaluate the potential key studies Chen et al. (2011) and Fawell et al. (1999) for use in the development of a RfD for microcystin-LR.

Current international guidelines or standards for microcystin-LR in drinking water are based on reported liver effects identified in the subchronic mouse study by Fawell et al. (1999). However, a new study (Chen et al., 2011) assessed reproductive effects in male mice following exposure to microcystin-LR in drinking water and identified sperm count and sperm motility as a sensitive toxicological endpoint.

#### **3.2.1 Are the methodologies of both studies sound? Please discuss the methodologies and their strengths and weaknesses.**

The methodologies of both studies appear to be sound. It is unfortunate that each one of them narrowed the focus of the study to only one system, reproductive or hepatic.

#### **3.2.2 Are strengths and weaknesses of each study appropriately described? Please provide suggestions, if needed.**

Many of the strengths and weaknesses of each study are described in the text of the report. I would also add:

1. Chen et al., 2011 – lack of replication of the male reproductive effects in other independent laboratories in other locations.
2. Chen et al., 2011 – Histopathology of testis – the descriptions are inadequate and do not provide sufficient detail to adequately assess the degree of damage.

#### **3.2.3 In this document, the Chen et al. (2011) is proposed as the critical study for developing the RfD. Please comment on this selection.**

### **8.1.1 RfD Determination**

#### **8.1.1.1 Choice of Key Study**

Pg 102. I disagree with the first sentence, “The key study for the development of an RfD for microcystins is that of Chen et al. (2011)...” because the EPA should not base an RfD on studies that have not been replicated at other independent laboratories at other locations regardless of how well those studies were performed at the first laboratory. Therefore, male reproductive effects should not be used as the endpoint. Hepatic effects have been widely shown and established at many different laboratories and should be used as the endpoint until such time as the use of other endpoints have been established.

#### **3.2.4 Please comment on the relative merits of Chen et al. (2011) vs Fawell et al. (1999) as the critical study. Which study represents the best available science and most appropriate toxicological endpoint for the basis of an oral RfD for microcystin-LR? Please provide the basis for your conclusion.**

Fawell represents the most appropriate toxicological endpoint and is the better choice because its endpoint and conclusions are supported by numerous studies from different labs around the world. In addition, the NOAEL level found in this study is based on a known concentration of MC-LR that was orally dosed to

the animals. Since the adoption of the WHO guideline of 1 ug microcystin /L in drinking water, toxicity at water concentrations of microcystins at or below 1 ug/L have not resulted in human or animal toxicity.

The effects on testis and spermatozoa reported in the Chen study have not been replicated and verified by other independent laboratories. Almost all of studies on the reproductive toxicity of MCLR come from this one laboratory and a small group of researchers in China over the past few years. Until these studies are replicated and confirmed in other laboratories, they should not be used to develop an oral RfD for microcystin-LR. In addition, since the water consumption of the mice in the study is not presented, the actual amount of MC-LR ingested by the mice is based on a calculation of the predicted water consumption of the mice in the study.

### **3.3 Calculation of RfD.**

This Health Effects Support Document proposes an oral RfD for microcystin-LR based on the sperm motility and sperm count effects identified in the Chen et al. (2011).

#### **3.3.1 Is the calculation of the RfD for microcystin-LR clear and accurate? Please discuss and provide suggestions, if needed.**

As far as I can assess it, however, this is not my area of specialty.

#### **3.3.2 Has uncertainty been adequately accounted for in the derivation of the RfD through the use of uncertainty factors? Please discuss and provide suggestions, if needed.**

As far as I can assess it, however, this is not my area of specialty.

#### **3.3.3 Specific Issues to address:**

##### **3.3.3.1-a The control group in Chen et al. (2011) did not receive any methanol to match the amount used to solubilize the microcystin-LR in the treated groups. Would treating the control and experimental group differently with methanol at the levels used in Chen et al. (2011) be anticipated to have an effect on the sperm count and motility? How does the lack of historical control data impact interpretation of the Chen data?**

Following a brief search, I could not find any studies which would indicate that this dosage of methanol would have an adverse reproductive effect. However, in general, I am concerned that the investigators did not use the appropriate negative control which would have been water with the same concentration of methanol in it.

##### **3.3.3.1-b Chen et al. (2011) did not provide data on testis weights. How does the lack of testis weights impact the interpretation of Chen et al. regarding the significance of the sperm effects? What is the impact on the strength and validity of the study if no information, or incomplete information was provided on how samples were handled and measurements were made (e.g., % sperm motility), mouse species, or the purity of MC-LR?**

There is no data on testis weights, nor on liver weights or lesions to confirm that the mice were being affected by the MC-LR. The data are incomplete and need to be replicated in other laboratories.

### **3.3.3.2 Are the sperm effects biologically plausible in humans?**

If humans have testicular OATps which can transport MC-LR into spermatogonia, Sertoli cells and/or Leydig cells, and if these cell types have protein phosphatases which bind to and are inhibited by MCLR, then yes, the sperm effects are biologically plausible in humans.

### **3.3.3.3 Would the male testicular effects reported by Chen et al. (2011) be anticipated to be reversible?**

Difficult to know for certain without further studies in which males were administered a chronic dose of MCLR for a defined number of days and then a group were allowed to recover from the MC-LR exposure. However, if type A spermatogonia are unaffected by MC-LR (which could be determined in a future study), then the effects might be reversible.

## **General Questions**

### **4. Is the document clear and understandable? Please describe and provide suggestions, if needed.**

a. Yes, it is.

### **5. Are you aware of any additional data that should be addressed in the document? If so, please provide a reference.**

Please see comments in previous sections.

### **6. Are you aware of any additional issues that should be addressed in the document?**

a. Abbreviations, pg xi, kg = kilogram

b. Chapter 4, p.19, 2<sup>nd</sup> paragraph, 2<sup>nd</sup> sentence: Should read, "In Lake Ontario, microcystin levels never exceeded 0.008 ug/L in the nearshore and were detected up to 0.076 ug/L in the bays and rivers. However, higher levels of microcystin, up to 1.6 to 10.7 ug/L, were found..." (Marakewicz, 2006).

c. Chapter 4, p. 22, 4.3.1 sentence beginning, "However, studies have reported that ingestion of cyanobacterial toxins may induce vomiting...(Puisseux-Dao and Edy, 2006)." This reference is on the use of Medaka fish in environmental toxicology. There are better references for the effects of cyanobacterial toxins on humans.

d. The Executive Summary should be updated to reflect any changes.

**COMMENTS SUBMITTED BY**

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**External Peer Review of the Draft *Health Effects Support Document (HESD)*  
for the Cyanobacterial Toxin Microcystins**

**Responses to Charge Questions from Dr. Jeanne M. Manson**

- 1. Chapters 2, 5, and 6** of the HESD provide information on the chemical and physical properties, exposure, and the toxicokinetics of microcystin.

**1.1 Are you aware of any additional data that should be included in the document? If so, please provide.**

I have conducted Medline and Google searches and have not identified any additional data that should be included in Chapter 2. Chapter 5 is beyond my technical expertise and I found the material difficult to read with often contradictory information. This Chapter could be reduced to emphasize areas where there are comparable data and consistent findings. Chapter 6 is well-written and the information appears to be complete.

**1.2 Is any of the information or conclusions included in the document incorrect, redundant or irrelevant? Please explain.**

Some of the information in Chapter 5 is irrelevant and consideration should be given to reducing this Chapter to emphasize areas where there are consistent findings. Chapter 6 is complete but some of the information is redundant. It would be sufficient to describe the consistent findings without citing numerous studies that came to the same conclusion.

**1.3 Please comment on the flow and continuity of these chapters and provide suggestions to enhance the utility of these chapters, if needed.**

Reduce the technical detail in Chapter 5 and 6 to improve continuity and flow.

**2. Chapter 7 - Hazard Identification.**

This chapter outlines toxicological studies, epidemiology, genotoxicity and mechanistic data. This chapter also includes the characterization of human health effects.

**2.1 Are you aware of any additional critical studies for microcystins that should be included in the document? If so, please provide.**

I have conducted independent literature searches and have not found any additional critical studies that should be included in the document.

**2.2 Is any of the information included in the document incorrect, redundant or irrelevant? Please describe and provide suggestions, if needed.**

There is information provided in this Chapter which may be considered redundant or irrelevant for hazard/risk assessment. The sections on protein phosphatase inhibition (p. 86), cytoskeletal disruption (p. 89), apoptosis (p.92) and reactive oxygen generation (p.94), while critical for hazard assessment, can be written in a much more concise manner.

**2.3 Are the conclusions and critical discussions for microcystins valid and scientifically defensible? Please describe and provide suggestions, if needed.**

Findings from the epidemiological studies are compelling, and more weight should be given to them, particularly the studies by Falconer et al. (1983) and Liu et al. (2011a). These investigators controlled for the temporal association between algal blooms and changes in liver enzymes (which is not possible in studies of colorectal cancer and hepatocellular carcinoma). The results were consistent across both studies and provide a strong rationale for liver toxicity with human exposure to environmentally relevant levels of microcystins.

The acute toxicity studies (oral, inhalation and dermal/ocular exposures) are well-described, as are the short term oral and inhalation studies. None of the subchronic studies reported changes in weight or histopathology of the testes.

**2.4 The work of Zhou et al. (2012) and Fardilha et al. (2013) are presented as supportive of a proposed *Mode of Action* for microcystin in its impacts on sperm cells in rats. Do these studies represent a reasonable mode of action for the effects seen in Chen et al. (2011)?**

In the description of the Chen et al. (2011) study (p. 69, end of first paragraph), the LOAEL is given as 0.79 mg/kg/day, and it should be 0.79 ug/kg/day. The lack of effect on testes weight in this study is notable given the severe testicular lesions and reduced sperm counts found. Otherwise, the studies by Zhou et al. (2012) and Fardilha et al. (2013) provide highly credible support for the proposed Mode of Action for microcystin.

**3. Chapter 8 - Dose-Response Assessment.**

This chapter provides the dose-response assessment and the derivation of RfD.

**3.1 Data sufficiency**

**3.1.1 Is the conclusion that there are sufficient data to derive a reference dose (RfD) for microcystin-LR adequately justified? Please discuss and provide suggestions, if needed.**

The conclusion that there are sufficient data to derive a RfD for microcystin-LR is well justified. Use of the Chen et al. (2011) study to derive a RfD is valid and supported by the data. Table 8.1 appears to be redundant to Table 7.13 as the same information is provided in both.

**3.1.2 Have critical data gaps been identified and/or addressed for cyanobacterial toxins? Please discuss and provide suggestions, if needed.**

I agree that use of methanol as a vehicle for microcystin-LR while water alone was used in the control group is not problematic and should not prevent use of this study for derivation of the RfD. I agree that much more information could have been presented on testes weight, but given the dimension of changes in other sperm parameters, this is not a fatal flaw. May investigators recommend that absolute testes weight be used rather than relative (to body weight) as the two parameters are not linearly related.

In the Falwell et al. (1999) 13-week study, all tissues from the control and high dose group were examined histopathologically, and lesions were found in the liver alone. The lack of lesions in the testes at the high dose group is notable and should be included as a critical data gap.

### **3.2 Identification of the critical study.**

Please critically review and evaluate the potential key studies Chen et al. (2011) and Fawell et al. (1999) for use in the development of a RfD for microcystin-LR.

See 3.1.1. The Chen et al. (2011) is the most appropriate study to use for the RfD; the lack of testicular findings in the high dose group of the Fawell et al. (1999) is a critical data gap.

Current international guidelines or standards for microcystin-LR in drinking water are based on reported liver effects identified in the subchronic mouse study by Fawell et al. (1999). However, a new study (Chen et al., 2011) assessed reproductive effects in male mice following exposure to microcystin-LR in drinking water and identified sperm count and sperm motility as a sensitive toxicological endpoint.

#### **3.2.1 Are the methodologies of both studies sound? Please discuss the methodologies and their strengths and weaknesses.**

See Sections 3.1.1 and 3.12 above.

#### **3.2.2 Are strengths and weaknesses of each study appropriately described? Please provide suggestions, if needed.**

The strengths and weaknesses are adequately described except for the lack of histopathological effects on the testes at the high dose in the 13 week study by Fawell et al. (1999).

#### **3.2.3 In this document, the Chen et al. (2011) is proposed as the critical study for developing the RfD. Please comment on this selection.**

I agree that the Chen et al. (2011) study is the most appropriate based on the route of exposure and quantification of sperm motility and sperm count.

#### **3.2.4 Please comment on the relative merits of Chen et al. (2011) vs Fawell et al. (1999) as the critical study. Which study represents the best available science and most appropriate toxicological endpoint for the basis of an oral RfD for microcystin-LR? Please provide the basis for your conclusion.**

See comments above. The Chen et al. (2011) study has the greatest relative merit and the sperm count and motility data should be used as the basis of an oral RfD.

### **3.3 Calculation of RfD.**

This Health Effects Support Document proposes an oral RfD for microcystin-LR based on the sperm motility and sperm count effects identified in the Chen et al. (2011).

#### **3.3.1 Is the calculation of the RfD for microcystin-LR clear and accurate? Please discuss and provide suggestions, if needed.**

I am not an expert in this area and therefore cannot evaluate the calculation of the RfD. The biological inputs to this model appear accurate to me.

**3.3.2 Has uncertainty been adequately accounted for in the derivation of the RfD through the use of uncertainty factors? Please discuss and provide suggestions, if needed.**

An uncertainty factor of 300 seems excessively high to me. I agree with the 10-fold factor for intraspecies extrapolation and the 3-fold for interspecies variability. The 10-fold factor for database insufficiencies appears arbitrary to me and this factor would more reliably be based on the quality of data available, which is high, rather than on missing data. A 3-fold factor seems more appropriate to me but these are subjective issues.

**3.3.3 Specific Issues to address:**

**3.3.3.1-a The control group in Chen et al. (2011) did not receive any methanol to match the amount used to solubilize the microcystin-LR in the treated groups. Would treating the control and experimental group differently with methanol at the levels used in Chen et al. (2011) be anticipated to have an effect on the sperm count and motility? How does the lack of historical control data impact interpretation of the Chen data?**

The issue of use of methanol as a vehicle in the treated but not the control group has already been addressed. The additional information provided by Chen et al. is highly reassuring that this is not a critical problem.

**3.3.3.1-b Chen et al. (2011) did not provide data on testis weights. How does the lack of testis weights impact the interpretation of Chen et al. regarding the significance of the sperm effects? What is the impact on the strength and validity of the study if no information, or incomplete information was provided on how samples were handled and measurements were made (e.g., % sperm motility), mouse species, or the purity of MC-LR?**

Chen et al. (2011) reported that there were no significant differences in testes weight between groups, which is different from saying testes weight data were not collected. I do not consider this to be a fatal flaw given the pronounced effects on sperm count and motility.

**3.3.3.2 Are the sperm effects biologically plausible in humans?**

It is highly likely they are biologically plausible for humans, as has been documented for interspecies extrapolation for agents such as cancer chemotherapy agents.

**3.3.3.3 Would the male testicular effects reported by Chen et al. (2011) be anticipated to be reversible?**

As has been well documented with dibromochloropropane (DBCP), it is anticipated that the male testicular effects would be reversible upon cessation of treatment. The exact time interval could be calculated if the testicular stage affected were known.

## **General Questions**

### **4. Is the document clear and understandable? Please describe and provide suggestions, if needed.**

The document is clear and understandable with the exception of redundancies in Chapters 5 and 7 described above. Overall, it is clear that a lot of hard work went into preparation of the document and it is technically strong.

### **5. Are you aware of any additional data that should be addressed in the document? If so, please provide a reference.**

I have performed independent literature searches and have not found any additional data that would be relevant to include

### **6. Are you aware of any additional issues that should be addressed in the document?**

No, the document is extremely thorough and the only issue is that parts of it can be reduced/summarized to make the information more readable.



**COMMENTS SUBMITTED BY**

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**External Peer Review of the Draft *Health Effects Support Document (HESD)*  
for the Cyanobacterial Toxin Microcystins**

**Responses to Charge Questions from Dr. Donald G. Stump**

- 1. Chapters 2, 5, and 6** of the HESD provide information on the chemical and physical properties, exposure, and the toxicokinetics of microcystin.

- 1.1 Are you aware of any additional data that should be included in the document? If so, please provide.**

I am unaware of any additional data that should be included in this document.

- 1.2 Is any of the information or conclusions included in the document incorrect, redundant or irrelevant? Please explain.**

Chapters 2, 5, and 6 are well written. I did not find any incorrect, redundant or irrelevant information.

- 1.3 Please comment on the flow and continuity of these chapters and provide suggestions to enhance the utility of these chapters, if needed.**

I thought that the chapters were well written and provide very good background information for the hazard identification and dose-response chapters.

**2. Chapter 7 - Hazard Identification.**

This chapter outlines toxicological studies, epidemiology, genotoxicity and mechanistic data. This chapter also includes the characterization of human health effects.

- 2.1 Are you aware of any additional critical studies for microcystins that should be included in the document? If so, please provide.**

I am unaware of any additional studies that should be included in this document.

- 2.2 Is any of the information included in the document incorrect, redundant or irrelevant? Please describe and provide suggestions, if needed.**

I have identified a few errors.

p.49, last sentence – The low dose should be 50 µg/kg/day, not 50 mg/kg/day.

p.56, 3<sup>rd</sup> sentence – The author states that that the mid and high dose groups had a trend towards higher FSH after 3 months which reached statistical significance by 6 months. This is only true for the high dose group. Statistical significance was not achieved in the mid dose group for FSH at 6 months.

p.56, 1<sup>st</sup> paragraph, last sentence – The LOAEL should be 0.79 µg/kg/day, not 0.79 mg/kg/day.

p.58, 3<sup>rd</sup> paragraph – The author does not list the route of administration in the description of the Li et al. study.

**2.3 Are the conclusions and critical discussions for microcystins valid and scientifically defensible? Please describe and provide suggestions, if needed.**

The authors have done a very thorough job of describing studies that are relevant for hazard identification of microcystins. I agree with the conclusions and believe the critical discussions are accurate based on the available data from the literature.

**2.4 The work of Zhou et al. (2012) and Fardilha et al. (2013) are presented as supportive of a proposed *Mode of Action* for microcystin in its impacts on sperm cells in rats. Do these studies represent a reasonable mode of action for the effects seen in Chen et al. (2011)?**

It is clear from the literature that microcystins require facilitated transport by OATp to enter cells. The manuscript from Zhou was able to demonstrate that using standard laboratory methods (isolation of spermatogonia, RT-PCR to measure OATp expression, Immunolabeling and Western blotting for determination of intracellular microcystin). The results clearly show that microcystins enter the spermatogonia and that OATp are present in the testis and spermatogonia. While Zhou did not demonstrate which OATp were responsible for facilitating transport of MC-LR into spermatogonia, previous studies have demonstrated that OATp are responsible for transport of MC-LR into the cell. Therefore, the data from the Zhou manuscript support the mode-of-action that microcystins negatively affect sperm cells in rats.

It is also clear from the literature that protein phosphorylation is critical for spermatozoa function. Fardilha was able to demonstrate that numerous protein phosphatases are present in human sperm. These phosphatases have been shown to be important for sperm motility, morphology and fertility. Inhibition of protein phosphatases has been shown to affect sperm motility. Although this manuscript did not investigate microcystins, previous studies have shown that MC-LR can inhibit protein phosphatases. Therefore, the data in this manuscript are supportive of the mode-of-action that microcystins can affect sperm cells through inhibition of protein phosphatases.

**3. Chapter 8 - Dose-Response Assessment.**

This chapter provides the dose-response assessment and the derivation of RfD.

**3.1 Data sufficiency**

**3.1.1 Is the conclusion that there are sufficient data to derive a reference dose (RfD) for microcystin-LR adequately justified? Please discuss and provide suggestions, if needed.**

While there are deficiencies with the reproductive studies that have been performed on microcystins, the authors have identified the deficiencies and I believe have correctly determined that sufficient data is available to derive a reference dose.

**3.1.2 Have critical data gaps been identified and/or addressed for cyanobacterial toxins? Please discuss and provide suggestions, if needed.**

The authors have done a good job of identifying the data gaps. I do not have any suggestions for additional data gaps.

### **3.2 Identification of the critical study.**

Please critically review and evaluate the potential key studies Chen et al. (2011) and Fawell et al. (1999) for use in the development of a RfD for microcystin-LR.

Current international guidelines or standards for microcystin-LR in drinking water are based on reported liver effects identified in the subchronic mouse study by Fawell et al. (1999). However, a new study (Chen et al., 2011) assessed reproductive effects in male mice following exposure to microcystin-LR in drinking water and identified sperm count and sperm motility as a sensitive toxicological endpoint.

#### **3.2.1 Are the methodologies of both studies sound? Please discuss the methodologies and their strengths and weaknesses.**

The Chen paper used sufficient sample sizes for data interpretation. Deficiencies include:

The authors do not present testes weights.

MC-LR was dissolved in methanol for the control group water did not contain any methanol.

The methodology used for sperm motility is completely lacking.

For sperm morphology, the sperm suspension was allowed to dry on the slide which can lead to artifacts. The preferred method is a wet-mount approach.

The fixation and staining of testes for microscopic examination were not optimal (Davidson's or Bouin's fixation followed by PAS staining).

Otherwise, the data is adequate. The sperm count data shows a clear dose-response at the mid and high doses groups that are more pronounced at 6 months than at 3 months. Similar effects were observed for sperm motility and hormone levels. The TUNEL assay also shows a clear dose-related increase in apoptotic cells at 6 months.

With regards to the Fawell manuscript, the 13-week study appears to be a routine toxicology study. Deficiencies include:

The methods are very sparse. It is a bit unclear as to what tissues were examined microscopically.

No body weight data is presented in the manuscript, only a summary in the text.

No data is presented for the developmental toxicity study. Therefore, I have no confidence that the authors' conclusions regarding this study are correct.

As long as we assume the 13-week study was performed according to standard practices, the conclusions drawn by Fawell (liver microscopic findings were observed in the mid and high dose group), are supported by the data.

**3.2.2 Are strengths and weaknesses of each study appropriately described? Please provide suggestions, if needed.**

The strengths and weaknesses of the Chen paper are assessed very thoroughly by the authors. I would suggest the authors add a couple more points for weaknesses to Section 8.1.1.2. The strain of mouse used in the study was not specified. Chen did not use best practices for microscopic examination of the testis (Davidson's or Bouin's fixation followed by PAS staining). In addition, a functional assessment of fertility (breeding to naive females) would greatly add to the value of the study.

The strengths and weaknesses of the Fawell manuscript relative to selection of the key study for RfD determination are adequately described.

**3.2.3 In this document, the Chen et al. (2011) is proposed as the critical study for developing the RfD. Please comment on this selection.**

I agree with the choice of the Chen study as the critical study because the dose level where effects were observed is much lower than the Fawell studies.

**3.2.4 Please comment on the relative merits of Chen et al. (2011) vs Fawell et al. (1999) as the critical study. Which study represents the best available science and most appropriate toxicological endpoint for the basis of an oral RfD for microcystin-LR? Please provide the basis for your conclusion.**

The Chen study is the critical study because it used the most appropriate route of administration (drinking water vs. gavage for the Fawell study) and the dose level where effects were observed (0.79 µg/kg/day vs. 200 µg/kg/day for the Fawell study). While some deficiencies have been identified in the Chen study as addressed in the EPA document and my peer review, these deficiencies are not enough to select the Fawell study as the critical study.

**3.3 Calculation of RfD.**

This Health Effects Support Document proposes an oral RfD for microcystin-LR based on the sperm motility and sperm count effects identified in the Chen et al. (2011).

**3.3.1 Is the calculation of the RfD for microcystin-LR clear and accurate? Please discuss and provide suggestions, if needed.**

The calculation of the RfD is clear and accurate.

**3.3.2 Has uncertainty been adequately accounted for in the derivation of the RfD through the use of uncertainty factors? Please discuss and provide suggestions, if needed.**

I agree with the use of 10x uncertainty factor for the deficiencies in the database. The Chen paper does not even specify the strain of mouse used. Other deficiencies have been noted by the authors and previously in my review. I agree with the interspecies 3x uncertainty factor because of toxicodynamic differences between mice and humans. Finally, I agree with the intraspecies 10x uncertainty factor for potential susceptible individuals in the human population.

### 3.3.3 Specific Issues to address:

#### 3.3.3.1-a **The control group in Chen et al. (2011) did not receive any methanol to match the amount used to solubilize the microcystin-LR in the treated groups. Would treating the control and experimental group differently with methanol at the levels used in Chen et al. (2011) be anticipated to have an effect on the sperm count and motility? How does the lack of historical control data impact interpretation of the Chen data?**

The low levels of methanol that were used to solubilize the MC-LR are not expected to affect sperm count and motility. With regards to sperm count, Chen states that the IVOS was used for the evaluation. This instrument has been used throughout the world for many years to assess sperm count. Sperm counts are very easy to perform. The magnitude of the difference from the control group is large (2- to 3-fold lower in the high dose group) and is dose-related. Therefore, the lack of historical control data is not a major concern for the sperm count data.

For the sperm motility data, the lack of information regarding the method used for motility assessment is of concern. I am also concerned about the long incubation period (1 hour). This long incubation period may explain why control motility values are lower than I am used to seeing. It would have been very helpful to see historical control data from the laboratory with regards to normal control motility values. In addition, progressive motility was not assessed. Therefore, I have less confidence in the motility data than in the count data. However, the motility data is very consistent with the sperm count data with regards to magnitude of difference versus control and the dose groups that are affected. While I have less confidence in the motility data, it does help support the use of sperm count as the most sensitive end point for risk assessment.

#### 3.3.3.1-b **Chen et al. (2011) did not provide data on testis weights. How does the lack of testis weights impact the interpretation of Chen et al. regarding the significance of the sperm effects? What is the impact on the strength and validity of the study if no information, or incomplete information was provided on how samples were handled and measurements were made (e.g., % sperm motility), mouse species, or the purity of MC-LR?**

At 6 months, Chen reports that sperm counts in the 10 µg/L group were more than 3-fold lower than controls. I would be very surprised if sperm count reduced to this extent did not affect testes weight. This assumption is further supported by the testicular histopathology effects in this group. However, the effects on histopathology and sperm count appear to be very strong. Therefore, I do not believe the study can be discounted. The lack of reporting the purity of MC-LR and strain of mouse are also problematic. Therefore, the addition of a 10x uncertainty factor is warranted.

#### 3.3.3.2 **Are the sperm effects biologically plausible in humans?**

Yes.

**3.3.3.3 Would the male testicular effects reported by Chen et al. (2011) be anticipated to be reversible?**

This is a difficult question to answer without assessing experimentally. In addition, I am not a pathologist making it difficult for me to draw conclusions on reversibility of the microscopic findings. As long as spermatogonia and Sertoli cells are still present, reversibility is a possibility.

**General Questions**

**4. Is the document clear and understandable? Please describe and provide suggestions, if needed.**

The document is clear, understandable and well written.

**5. Are you aware of any additional data that should be addressed in the document? If so, please provide a reference.**

I am not aware of any additional data that should be addressed.

**6. Are you aware of any additional issues that should be addressed in the document?**

On p.103, there are 2 typographical errors. In the second full sentence “evident” is spelled incorrectly. In the second sentence of the second paragraph, “corresponding” is spelled incorrectly.

**COMMENTS SUBMITTED BY**

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**External Peer Review of the Draft *Health Effects Support Document (HESD)*  
for the Cyanobacterial Toxin Microcystins**

**Responses to Charge Questions from Dr. Xiaozhong Yu**

- 1. Chapters 2, 5, and 6** of the HESD provide information on the chemical and physical properties, exposure, and the toxicokinetics of microcystin.

**1.1 Are you aware of any additional data that should be included in the document? If so, please provide.**

HESD has compiled all available information on the chemical and physical properties, exposure, and the toxicokinetics of microcystin. No additional data were found during the review period.

**1.2 Is any of the information or conclusions included in the document incorrect, redundant or irrelevant? Please explain.**

Page 6, Line 1 Table 0-2 should be Table 2-2

**1.3 Please comment on the flow and continuity of these chapters and provide suggestions to enhance the utility of these chapters, if needed.**

Page 11, Line 18 “In marine systems, salinity gradients also induce stratification. As temperatures rise due to climate change, waters are expected to stratify earlier in the spring and the stratification will persist longer into the fall (Paerl and Otten, 2013b).”. It is unclear what is the purpose of these sentences. There is no evidence to directly support that these changes have anything important to the microcystin.

Page 27, Line 5 “In children, they have been used as an alternative, natural therapy to treat attention deficit hyperactivity disorders (ADHD).” It is unclear to me the rationale to mention the children and talk about ADHD. Is it related to the risk assessment of MC-LR?

**2. Chapter 7 - Hazard Identification.**

This chapter outlines toxicological studies, epidemiology, genotoxicity and mechanistic data. This chapter also includes the characterization of human health effects.

**2.1 Are you aware of any additional critical studies for microcystins that should be included in the document? If so, please provide.**

**Page 58, Line 12 of the paragraph 2** “Histologically both treatment groups had atrophy of the seminiferous tubules with increased spacing between the seminiferous tubule cells. The effect increased with increasing dose. The high-dose group also exhibited deformation of androgonial and sperm mother cells, and decreased number of interstitial cells, Sertoli cells, and mature sperm in the seminiferous tubules.” It is unclear what is “androgonial and sperm mother cells”. It should be explained using the updated terminology.

**2.2 Is any of the information included in the document incorrect, redundant or irrelevant? Please describe and provide suggestions, if needed.**

**2.3 Are the conclusions and critical discussions for microcystins valid and scientifically defensible? Please describe and provide suggestions, if needed.**

Page 89, Line 27 “A study in China evaluated liver damage in children in relation to the microcystin levels in the drinking water and select aquatic foods (e.g. carp and duck) (Li et al., 2011a). Microcystin levels were associated with increasing levels of AST and ALP, but not ALT and GGT. The OR for liver damage as reflected by increased serum enzyme levels in exposed children was 1.72 (95% CI: 105-2.76).” I would like to suggest to use “increasing level of AST and ALP” instead of “liver damage”. Increase of ALP or AST does not mean the damage of the liver. Other diseases or factors can also cause the increase of ALP. The normal range of male and female is significantly different, males from 14-20 U/L, and female from 10 to 36 U/L. The results of this study did not separate the gender. It seems that all the values for the AST and ALP were within the normal range. Therefore, it is inappropriate to conclude that microcystin exposure led to liver damage.

Page 90, Line 30 “Evidence for effects of MC-LR on the male reproductive system and sperm development following oral exposures were reported by Chen et al. (2011) and are supported by i.p. data (Liu et al., 2010; Chen et al., 2013). Oral exposure and i.p treatment are significantly different, especially for microcystin. Microcystin-LR is  $30 \pm 100$  times less toxic via oral ingestion than via intraperitoneal injection (Fawell et al., 1999). The changes of male reproductive functions in i.p. did not support that oral exposure at low concentration could also result in dysfunction of the male reproductive system and sperm development. It should be revised.

Page 90, Line 36, “deformation of androgonial and sperm mother cells;” should be updated using most recent terminology.

Page 91, Line 3, it should add that microcystin (MC-LR) affects hormones level of male mice by damaging hypothalamic-pituitary system, but MC-LR was not able to enter Leydig cells and had no cytotoxicity on Leydig cells in vivo test. These results suggested that MC-LR affected male mice serum hormones and mRNA expressions by damaging the hypothalamic-pituitary systems (Wang et al., 2011).

Page 91 Line 4, It is really too sudden to follow the paragraph “The concerted action of protein phosphatases and kinases regulating the phosphorylation of the cytoskeleton is known to be important to sperm physiology. In a study of human normozoospermic and asthenozoospermic samples, Fardilha et al. (2013) identified a significant decrease in the cellular distribution of the PP1 and PP2 subfamilies that correlated with the low motility for the asthenozoospermic samples. The progressive motility of sperm in the asthenozoospermic samples was about 10% of that for the normal sperm and the number of immotile sperm was about twice that for the normal samples. Fardilha et al. (2013) is not a study of microcystins but gives credibility to the hypothesis that inhibition of protein phosphatases can adversely impact sperm motility.” There was no any paragraph discussing “that inhibition of protein phosphatases” is the mechanism of MC-LR induced-dysfunction of the sperms. There was no any discussion of the hypothesis. It is weird that this cited paper gave the credibility of the hypothesis. It is very critical to make clear what is the hypothesis, and who proposed the hypothesis.

Page 91 Line 16 “Observed effects in *in vivo* studies include decreased sperm motility, viability, and counts; reduced spermatogonia and spermatid quality; and increases in abnormal sperm (Ding et al., 2006; Li et al., 2008). **Numerous** histological changes have also been observed in the testes including testicular atrophy and degeneration; depopulation of the Leydig, Sertoli, and mature sperm cells; and increased apoptosis (Ding et al., 2006; Li et al., 2008).” Reduced spermatogonia was not a significant change. In fact, there was no very robust study to demonstrate it. “Numerous histological changes” were not well justified by reviewing the publication. The most frequent histopathological observations were suffering from the artifacts of the fixation of the testis. Increased empty spaces between the seminiferous tubules were observed in the most of the histopathological examinations (Chen et al., 2013). Unfortunately, it was also observed in the most of the control from the representative photos since it was due to the inappropriate fixation of the testis tissue. It seems that the decrease of the Leydig cells was consistently observed, but no robust quantitative analysis to support it.

Page 92, Line 7 from bottom, “The damage observed in each of the tissues impacted by microcystins (liver, testes, kidney, etc.) can be correlated with the mode of action events described above. The adverse effects observed are consistent with the postulated mode of action as are the dose-related increases in effect severity” Please describe clearly what is the “mechanistic mode of action of MC-LR induced adverse effects”. Simply listing of the reported changes of OATp transporter, phosphatase inhibition, cytoskeleton or the generation of ROS does not guarantee these observed changes are the MOA of MC-LR induced adverse effects in the target tissue of the liver or male reproductive systems. Again, it is unclear to me what is the postulated model of action.

Page 98, Line 1 to 5, “Available information does not suggest any pronounced gender differences in response to microcystins for the liver. Studies with cyanobacterial extracts suggest the possibility that male mice may be more sensitive than female mice to oral exposure to cyanobacterial extracts (Falconer et al., 1988). There are gender differences for reproductive effects as a consequence of sperm count, sperm motility, abnormal sperm, and histological alterations observed in the testes.” The above conclusions are misleading. Studies with cyanobacterial extracts were focusing on the male reproductive system. There were very few studies focusing on the effect on the female reproductive system. Based on the consistent effects of the hormonal changes (FSH, LH) and potential targets on hypothalamic-pituitary systems, exposure to female animals might result in changes of estrous cycle, and ovulation. There is a lack of information regarding the sensitivity in the reproductive system. The majority of the studies published in the male reproductive system do not mean that male reproductive system is more sensitive than the female reproductive system.

#### **2.4 The work of Zhou et al. (2012) and Fardilha et al. (2013) are presented as supportive of a proposed *Mode of Action* for microcystin in its impacts on sperm cells in rats. Do these studies represent a reasonable mode of action for the effects seen in Chen et al. (2011)?**

I do not believe the work of Zhou et al. (2012) and Fardilha et al. (2013) are supportive of a proposed MOA for MC-LR in its impact on sperm cells in rats, and strongly oppose this conclusion. First, despite the adverse effect of MC-LR were observed in the male reproductive system, the target tissue or cells are still unclear. It has never been demonstrated that MC-LR can pass the testis-blood barrier and reach to the seminiferous tubes, to the germ cells including spermatogonia, Sertoli cell or Leydig cells. Although it was listed as one of the goal to measure the MC-LR level in the testis and epididymis by LC-MS in Chen et

al., 2011 paper, no result was shown in the paper, and even no discussion of it. Wang et al., 2012 revealed that MC-LR by intraperitoneal injection induced significant decrease in the GnRh expression in a dose- and duration-dependent manner. The serum LH and testosterone exhibited similar trends of change, with both LH and testosterone increased in 30  $\mu\text{g kg b.w.}_1 \text{ day}_1$  group after 1 day. And 15  $\mu\text{g kg b.w.}_1 \text{ day}_1$  group increased also after 4 days. But after 7 days 30  $\mu\text{g kg b.w.}_1 \text{ day}_1$  group fell to control level. While after 14 days, compared to control group, in all concentration-groups both of them decreased significantly. Furthermore, in vitro Leydig cell culture demonstrated that there was no uptake of MC-LR, consistent with the no cytotoxicity of Leydig cells. The results from this study showed that MC-LR affected male mice serum hormones and mRNA GnRh expressions by damaging the hypothalamic-pituitary systems. Second, although various histopathological changes have been reported in the testis, no convincing evidence showing the target cells. The most widely reported changes of the testis were the increase of the empty spaces between the seminiferous tubes. However, as evident from the representative photos from the control animals, there were empty spaces too. The majority of the studies did not use the recommended fixation for the testis because the routine histopathological approach can not preserve the unique structure of the testis. Histopathological evaluation of the testis could provide one of the most sensitive end points for detecting the effects of toxicants. It is routinely applied in the evaluation of male reproductive toxicity. However, "routine" histological such as paraformaldehyde based fixation methods are often inadequate for maintaining the "sensitivity" of this type of evaluation. Improper fixation and inappropriate combinations of fixative and embedding media result in unacceptable histological sections (1). The distortions induced by inadequate methods can make the detection of differences between treated and control tissues nearly impossible at all. As stated in the book chapter 4 by Hess and Moore "Formalin alone should never be used to fix testes to be embedded in paraffin. The best results are obtained in paraffin, using either Bouin's fixative or a primary fixation in neutral buffered formalin (NBF) followed by Bouin's fixative. The benefit of the dual fixation is that the tissues also appear well fixed in GMA medium; therefore, if quantitative data are needed subsequent to a general evaluation of paraffin sections". (Histological Methods for Evaluation of the Testis, Rex A. Hess and Billy J. Moore in METHODS IN TOXICOLOGY, Volume 3A). In order to assure the result from the experiment with testis, it is highly recommended to apply the guideline developed by the reproductive expert panel, "Recommended Approaches for the Evaluation of Testicular and Epididymal Toxicity" TOXICOLOGIC PATHOLOGY, vol 30, no 4, pp 507–520, 2002. The fixation methods for the testis is widely recommended to use Bouin's-solution in order to preserve the microstructure of testis. Sections are recommended to stain with the Periodic acid Schiff (PAS) technique and counter-stained with hematoxylin. The fixation with 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) (pH = 7.4) will generate a lot of artifacts, such as the loosen of the testicular tubes. So far, there is no evidence that treatment of MC-LR target the spermatogonia and lead to the depletion of the spermatogonia in the seminiferous tubes (I have reviewed all the photos of the cross-section of testis published). Liu et al., 2010 reported that lesions such as changes in both spermatogonia and Sertoli cells were seen in animals treated with 12.5  $\mu\text{g MC-LR equivalents/kg}$ . But Liu et al., also claimed that recovery occurred by 48 hours with the tissue resembling the control (Liu et al., 2010). Spermatogonia cells are undifferentiated male germ cell, originating in a seminiferous tubule and dividing into two primary spermatocytes in the production of spermatozoa. Damage or reduction of the pool of spermatogonia cells will result in a decrease of the other type of germ cells. It is very hard to understand that the damage of spermatogonia cells would be recovered within 48 hours. Increased empty spaces or loosened microstructure between the seminiferous

tubes suggested that MC-LR might target the Leydig cells, which eventual lead to the decrease of the testosterone level. But the increase of the empty spaces also could be the defects from the testis fixation. Therefore, the application of the in vitro culture of spermatogonia to examine the potential mechanism is questionable. The existence of nontransporting polypeptides (Oatps) in the spermatogonia necessary means that MC-LR could enter into spermatogonia since the in MC-LR has to first pass the blood testis barrier. Also the in vitro observation of uptake of MC-LR by the spermatogonia does not mean uptake in vivo. So far it is very clear that spermatogonia is not the target cells of the MC-LR, therefore, Zhou et al. (2012)'s paper could not provide direct information of the Mode of action for the MC-LR induced adverse effects in the testis.

Fardilha et al. (2013) reported an important research on the protein phosphatases (PPs) of the human sperms, and identified three new serine/threonine-protein PPs, PPP1CB, PPP4C, and PPP6C together with two tyrosine-PPs, MKP1 and PTP1C. It is reasonable to infer from the finding of Fardilha et al. (2013) that inhibition of protein phosphatases can adversely impact sperm motility. But it does not mean that MC-LR can inhibit the activities of these phosphatases. It might be true MC-LR could inhibit those PPs, but in fact, there was no study reporting MC-LR inhibit the human sperm motility through the inhibition of PPs. That "inhibition of protein phosphatases" is the Mode of Action of MC-LR induced-dysfunction of the sperms needs to be further verified. We need to verify the target tissue or target cells of the MC-LR induced male dysfunction. We need to verify whether the decrease of the sperm count or sperm motility is due to the damage of the testis, or due to the damage of testis such as the depletion of spermatogonia in the testis or due to the depletion of the Leydig cells leading to the decrease of the testosterone. We still need to verify whether MC-LR directly inhibits the PPs in the epididymis and impairs the sperm development. We still need to verify whether the MC-LR directly damage the hypothalamic-pituitary systems (Wang et al., 2011), and adverse effect on the sperm count and motility were the secondary effects of the changes of hormones such as FSH, LH and testosterone. Although the paper is very informative and probably imply potential explanation, so far there is no direct evidence supporting that inhibition of PPS is the mechanism of MC-LR induced malformation or decreased count of sperm. Therefore, I do not think Fardilha et al. (2013) studies represent a reasonable mode of action for the effects seen in Chen et al. (2011)?

### **3. Chapter 8 - Dose-Response Assessment.**

This chapter provides the dose-response assessment and the derivation of RfD.

#### **3.1 Data sufficiency**

##### **3.1.1 Is the conclusion that there are sufficient data to derive a reference dose (RfD) for microcystin-LR adequately justified? Please discuss and provide suggestions, if needed..**

Due to the concern of the data quality in Chen et al., 2011, as reflected in the incompleteness of the study design, unknown strain of mouse and ages, deficiency in the description of method used in sperm count and sperm motility analysis, inappropriate fixation approach in the histopathological examination of testis, and staining of the cross-section, and descriptive observation of the morphological examination of the histology, it is very hard to justify to use dataset without quality insurance. The dose-response data from Fawell et al., 1999, can be used to derive a RfD for MC-LR.

**3.1.2 Have critical data gaps been identified and/or addressed for cyanobacterial toxins? Please discuss and provide suggestions, if needed.**

Please see comments in 2.4.

1. It is unclear whether the “inhibition of protein phosphatases” is the Mode of Action of MC-LR induced-dysfunction of the sperms.
2. There is a need to verify the target tissue or target cells of the MC-LR induced male reproductive dysfunction.
3. There is a need to verify whether the decrease of the sperm count or sperm motility is due to the damage of the testis, or due to the damage of testis such as the depletion of spermatogonia in the testis or due to the depletion of the Leydig cells leading to the decrease of the testosterone.
4. There is a need to verify whether MC-LR directly inhibits the PPs in the epididymis and impairs the sperm development.
5. There is a need to verify whether the MC-LR directly damage the hypothalamic-pituitary systems (Wang et al., 2011), and adverse effect on the sperm count and motility were the secondary effects of the changes of hormones such as FSH, LH and testosterone.

**3.2 Identification of the critical study.**

Please critically review and evaluate the potential key studies Chen et al. (2011) and Fawell et al. (1999) for use in the development of a RfD for microcystin-LR.

Current international guidelines or standards for microcystin-LR in drinking water are based on reported liver effects identified in the subchronic mouse study by Fawell et al. (1999). However, a new study (Chen et al., 2011) assessed reproductive effects in male mice following exposure to microcystin-LR in drinking water and identified sperm count and sperm motility as a sensitive toxicological endpoint.

**3.2.1 Are the methodologies of both studies sound? Please discuss the methodologies and their strengths and weaknesses.**

Regarding the publication of Chen et al., 2011, it is a critical publication regarding the potential effects of microcystin-LR on the male reproductive system. However, the methodology and analysis used in the manuscript lead me to concern about the reliability of the results.

The followings are the detailed problems observed in this paper.

**1. Study design**

As illustrated in the Figure 1, LC-MS is proposed to measure the concentration of microcystin-LR (MC-LR) in epididymis of 10 mice, and testis of 15 mice, but these data never mentioned in the results. These data will be critical to evaluate the testicular toxicity since it is still unclear whether the MC-LR could pass the Testis-blood barrier, and whether MC-LR distribute to the epididymis.

It could be negative or positive results from these LC-MS measurements. However, no mention of LC-MS result in the result section or even no mention in the discussion reflected the quality of the research work. At least, this publication was not a high quality research!

Page 552 “Of 20 mice in each group, the right epididymides from 10 mice were used to carry out the sperm quality and the left 10 epididymides were saved to check the quantitative of MC-LR by LC-MS. Because the volume of serum was limited, all the blood samples in our study were double-diluted. The 10 samples were chosen from 40 samples at random and represented 10 different mice. Five testes from 5 mice were used for histopathological analysis and TUNEL staining. The remaining 15 mice were used for qualitative and quantitative analysis of testicular MC-LR by LC-MS (Fig. 1).”

## 2. Mice, strain and ages

There was no information about the strain of the mice. The strain difference in response to chemical treatment is reported to MC-LR. The age of these mice is unclear. Based on the body weight information stated in the paper from 15 to 25 g, and it is assumed the strain of mice is BALB/c, the age of these mice might be between 3 weeks to 8 weeks. There are a huge difference of development of male reproductive system in ages, and response to chemicals is different.

## 3. Sperm Analysis

There is a lack of information about the sperm analysis. “It was minced into 1-mm pieces and incubated in 2mL BWW medium at 32 °C for 1 h. Sperm counts were determined through an automatic semen analyzer (VERSION 12.2, HTM-TOX IVOS).”

Since the measurement of sperm analysis was carried through the computer-assisted sperm analysis (CASA). There is no description of the analytic protocol. Neither the information about the quantitative parameters of sperm motility obtained from the CASA. The traditional manual examination of sperm count and motility measurement under the microscope is quite subjective; therefore, it is emphasized that the operator should be blinded. However, the importance to declare “This operation was performed by an operator who was blind to the group assignment of animals” is unclear. The lack of description of the analytic protocol as well as the sub-professional statement leads to the concern of the quality of the results.

A normal description of CASA from the HTM-IVOS Sperm Analyzer during measurements normally includes the parameters such as minimum contrast, minimum cell size, straightness threshold, path velocity cutoff, progressive minimum path velocity, static head size, static head intensity, and static elongation. The calculation of motility of the sperm is unclear. HTM-TOX IVO (version 12), a computer-assistant sperm analysis (CASA), routinely provides the following information including Total, Static Progressive, Motile, Slow, Bent head, Coiled tail, Distal droplet, and Proximal droplet. Therefore, it can be concluded that the sperm analysis in this paper was not carried out with quality insurance.

#### 4. Serum hormone assay

As described in the Chen 2011 paper, “the blood samples were taken from the eye”, it is practically impossible to collect 1 ml from the eye. Generally speaking, in Balb mice, the blood accounts 0.04-0.06 ml of BW, or 1.0-1.5 ml blood from a 25 gm mouse. The best yields are obtained if the blood is removed slowly and steadily so that the heart is kept beating as long as possible. There is no description of the protocol for the collection of blood from the eye. Is the mouse under the anesthesia procedure? Therefore, this is another example, raising the concern about the accuracy of the data handling, and quality insurance. As stated in the paper, “Because the volume of serum was limited, all the blood samples in our study were double-diluted”, however, it is unclear how these samples diluted. What is the procedure call “double diluted” ? Is the blood sample or serum diluted? What solution is used to dilute?

The results shown in Figure 3 A and B were mean  $\pm$ S.E. The standard deviation for the serum testosterone for the control and the groups without statistical significance are huge, but all the groups with statistic significance were very small. The variation of the testosterone in the control is consistent with the publication, however, the physiological implication of the significant decrease in the S.E. in the high dose groups is unclear, and have not discussed.

#### 5. Histopathological evaluation

Histopathological evaluation of the testis provides one of the most sensitive end points for detecting the effects of toxicants. It is routinely applied in the evaluation of male reproductive toxicity. However, "routine" histological such as paraformaldehyde based fixation methods are often inadequate for maintaining the "sensitivity" of this type of evaluation. Improper fixation and inappropriate combinations of fixative and embedding media result in unacceptable histological sections (1). The distortions induced by inadequate methods can make the detection of differences between treated and control tissues nearly impossible at all. As stated in the book chapter 4 by Hess and Moore “Formalin alone should never be used to fix testes to be embedded in paraffin. The best results are obtained in paraffin, using either Bouin's fixative or a primary fixation in neutral buffered formalin (NBF) followed by Bouin's fixative. The benefit of the dual fixation is that the tissues also appear well fixed in GMA medium; therefore, if quantitative data are needed subsequent to a general evaluation of paraffin sections” (Histological Methods for Evaluation of the Testis Rex A. Hess and Billy J. Moore in METHODS IN TOXICOLOGY, Volume 3A).

It is highly recommended to apply the guideline developed by the reproductive expert panel, “Recommended Approaches for the Evaluation of Testicular and Epididymal Toxicity” TOXICOLOGIC PATHOLOGY, vol 30, no 4, pp 507–520, 2002. The fixation methods for the testis is widely recommended to use Bouin's-solution in order to preserve the microstructure of testis. Sections are recommended to stain with the Periodic acid Schiff (PAS) technique and counter-stained with hematoxylin. The fixation with 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) (pH = 7.4) will generate a lot of artifacts, such as the loosen of the testicular tube.

Spermatogenesis is a cyclic process during which, within each epithelial area, various generations of germ cells undergo a series of developmental steps according to a fixed time schedule. The cycle of the seminiferous epithelium can be subdivided into stages. In the mouse, 12 such stages have been described that can be distinguished from one another by steps in spermatid development. In order to compare the

effect of chemicals on the spermatogenesis, a careful examination of the stage, counting of different cells in each stage is critical to pinpoint the potential effect on the testis. One example to evaluate the pathological changes in testis can be found in *Toxicology and Applied Pharmacology* 174, 35–48 (2001). Quantification of the cells in different stage of the tubule is listed.

As listed in Figure 4 A, it was claimed that “No significant difference in the spermatogenic epithelium in seminiferous tubules was observed between control and MC-LR treated groups”. Even with a well-experienced histopathological expert, it will be very hard to judge through these representative photos. As stated previously, the paraformaldehyde fixation did not preserve the fine microstructure. Cytoplasmic shrinkage and chromatin aggregations were observed in control and treatment group. Loosen structure leading to numerous empty spaces between cells were observed in all groups.

Without quantitative evaluation of the cells in the different stage of the tubules, it is very hard to tell the difference of seminiferous tubes. It is unclear how the author concluded “In comparison with control, the spermatogenic epithelium became sparse at 3.2  $\mu\text{g/L}$ . The structure of the spermatogenic epithelium was at a loss, deranged and thinner at 10  $\mu\text{g/L}$  of MC-LR.” It is unclear the authors concluded that “the  $\square\square\square\mu\text{g/L}$  group also showed a loss and derangement of spermatogenic cells, enlargement of the lumen of the seminiferous tubules, thinning of the spermatogenic epithelium (Fig. 4B-d)”. Especially, how the authors concluded that MS-LR treatment lead to “depopulation of Leydig cells, Sertoli cells, and mature sperm” since these pictures did not show clearly where is the Sertoli cells or Leydig cells in the control. Again, these less-professional evaluation of the histopathological changes significantly compromised the quality of the research, and therefore, it need to take serious concern of the result.

## 6. TUNEL Cell counts

“The number of the testicular cells in sections which were positive for TUNEL (green) was counted from 10 fields, selected at random and observed under the fluorescence microscope (X400)”. It is very curious how to randomly select 10 fields under microscope. The examiner has to move the stage and observe the microscope field and adjust the focus. This procedure is very subjective and not a random procedure. How the percentage of the TUNEL positive cells are calculated is not described in the paper. Assuming the results in Figure 5 C and 5D are right, then the representative figures in 5A and 5B are misleading. There were no apoptotic cells in Fig 5Aa, b or c. It is also unclear what is the cell type of these apoptotic cells. In summary, due to the lack of detailed information about the protocol used to collect the data, inappropriate methodology used to fix the testis tissue, and the lack of objective and quantitative evaluation of the morphology, the quality of the research is compromised, and the results are not reliable. Therefore, it needs to take an additional cautious to use these data in the risk assessment.

### **3.2.2 Are strengths and weaknesses of each study appropriately described? Please provide suggestions, if needed.**

See comments in 3.2.1.

**3.2.3 In this document, the Chen et al. (2011) is proposed as the critical study for developing the RfD. Please comment on this selection.**

See comments in 3.2.1.

*Point of clarification:*

I have clearly given my answers in the review report. The methodology, and description of the protocol and the evaluation of the results were questionable, therefore, it is very hard to defend the data quality. I still believe the data from Fawell et al. 1999 are more defensive than Chen's.

**3.2.4 Please comment on the relative merits of Chen et al. (2011) vs Fawell et al. (1999) as the critical study. Which study represents the best available science and most appropriate toxicological endpoint for the basis of an oral RfD for microcystin-LR? Please provide the basis for your conclusion.**

See comments in 3.2.1.

**3.3 Calculation of RfD.**

This Health Effects Support Document proposes an oral RfD for microcystin-LR based on the sperm motility and sperm count effects identified in the Chen et al. (2011).

**3.3.1 Is the calculation of the RfD for microcystin-LR clear and accurate? Please discuss and provide suggestions, if needed.**

Yes.

**3.3.2 Has uncertainty been adequately accounted for in the derivation of the RfD through the use of uncertainty factors? Please discuss and provide suggestions, if needed.**

Yes.

**3.3.3 Specific Issues to address:**

**3.3.3.1-a The control group in Chen et al. (2011) did not receive any methanol to match the amount used to solubilize the microcystin-LR in the treated groups. Would treating the control and experimental group differently with methanol at the levels used in Chen et al. (2011) be anticipated to have an effect on the sperm count and motility? How does the lack of historical control data impact interpretation of the Chen data?**

See other comments in 3.2.1

**3.3.3.1-b Chen et al. (2011) did not provide data on testis weights. How does the lack of testis weights impact the interpretation of Chen et al. regarding the significance of the sperm effects? What is the impact on the strength and validity of the study if no information, or incomplete information was provided on how samples were handled and measurements were made (e.g., % sperm motility), mouse species, or the purity of MC-LR?**

Lack of testis weight data, detailed information about the protocol used to collect the sperm count and motility data, inappropriate methodology used to fix the testis tissue, and the lack of objective and

quantitative evaluation of the morphology, the quality of the research is severely compromised, and the results are not reliable. The incomplete information just reflected how the samples were handled and measurements were made (e.g., % sperm motility). Therefore, it needs to take an additional cautious to use these data in the risk assessment. **Detailed protocol of sperm count See other comments in 3.2.1**

### **3.3.3.2 Are the sperm effects biologically plausible in humans?**

There is no human data on the adverse male reproductive function induced by MC-LR reported so far, even under high exposure levels. It is unclear whether oral exposure to MC-LR could accumulate in the testis or epididymis. At least whether MC-LR inhibit those PPs and impair the human sperm motility is unclear. It is very hard to conclude MC-LR induced effects on animals at low dose are biologically plausible in human.

### **3.3.3.3 Would the male testicular effects reported by Chen et al. (2011) be anticipated to be reversible?**

Based on the morphological examination of the testis, and also one study in rabbit testis, the MC-LR effect on sperm count or motility is reversible. However, as discussed previously, the target organ, tissue or cells are still unclear so far. If the effect of the MC-LR on the male reproductive system is the secondary effect from the damage of the hypothalamic-pituitary systems (Wang et al., 2011), then it is very hard to tell whether the adverse effects on male reproductive system is reversible or irreversible.

## **General Questions**

### **4. Is the document clear and understandable? Please describe and provide suggestions, if needed.**

To some extent. Please refer other comments.

### **5. Are you aware of any additional data that should be addressed in the document? If so, please provide a reference.**

None.

### **6. Are you aware of any additional issues that should be addressed in the document?**

See all other comments.